COMPARISON OF ACTIVITIES, ELECTROPHORETIC PATTERNS AND KINETIC PROPERTIES OF SOME ENZYMES IN NORMAL AND CANCEROUS LIVER

Thesis presented in fulfillment of the requirements for

the degree of Doctor of Philosophy

at the University of the Witwatersrand

by

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DECLARATION

I hereby declare that the work forming the basis of this thesis is my own and has not previously been submitted for any other degree at any University.

K. D. Hammond

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INTRODUCTION

ENZYMES OF CARBOHYDRATE METABOLISM IN NORMAL AND TUMOUR TISSUES AND CELL CULTURES

Glucose present in tissues is metabolised either by means of glycolysis, gluconeogenesis, glycogenesis or glycogenolysis, as shown in Figs. 1 and 2. Glycolysis occurs in almost all tissues, whereas gluconeogenesis is restricted mainly to liver and kidney cortex; the synthesis and degradation of glycogen takes place mainly in liver, skeletal muscle and heart.

The patterns of carbohydrate metabolism characteristic of normal tissue are often altered in various diseased states; this is particularly evident in cancer. Tumour tissues, such as hepatoma, appear to lose the functional properties of adult differentiated tissue and their metabolism often seems to resemble that of rapidly-dividing, undifferentiated foetal tissue.

Activities of enzymes in livers, hepatomas and cell cultures

General

An important function of normal adult liver is the control of carbohydrate metabolism by means of glycolysis, gluconeogenesis and glycogen synthesis and degradation. The overall rates of these processes are determined by the activities of the key rate-limiting enzymes: hexokinase, phosphofructokinase and pyruvate kinase (glycolysis), pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-diphosphatase and glucose-6-phosphatase (gluconeogenesis), glycogen synthetase (glycogen

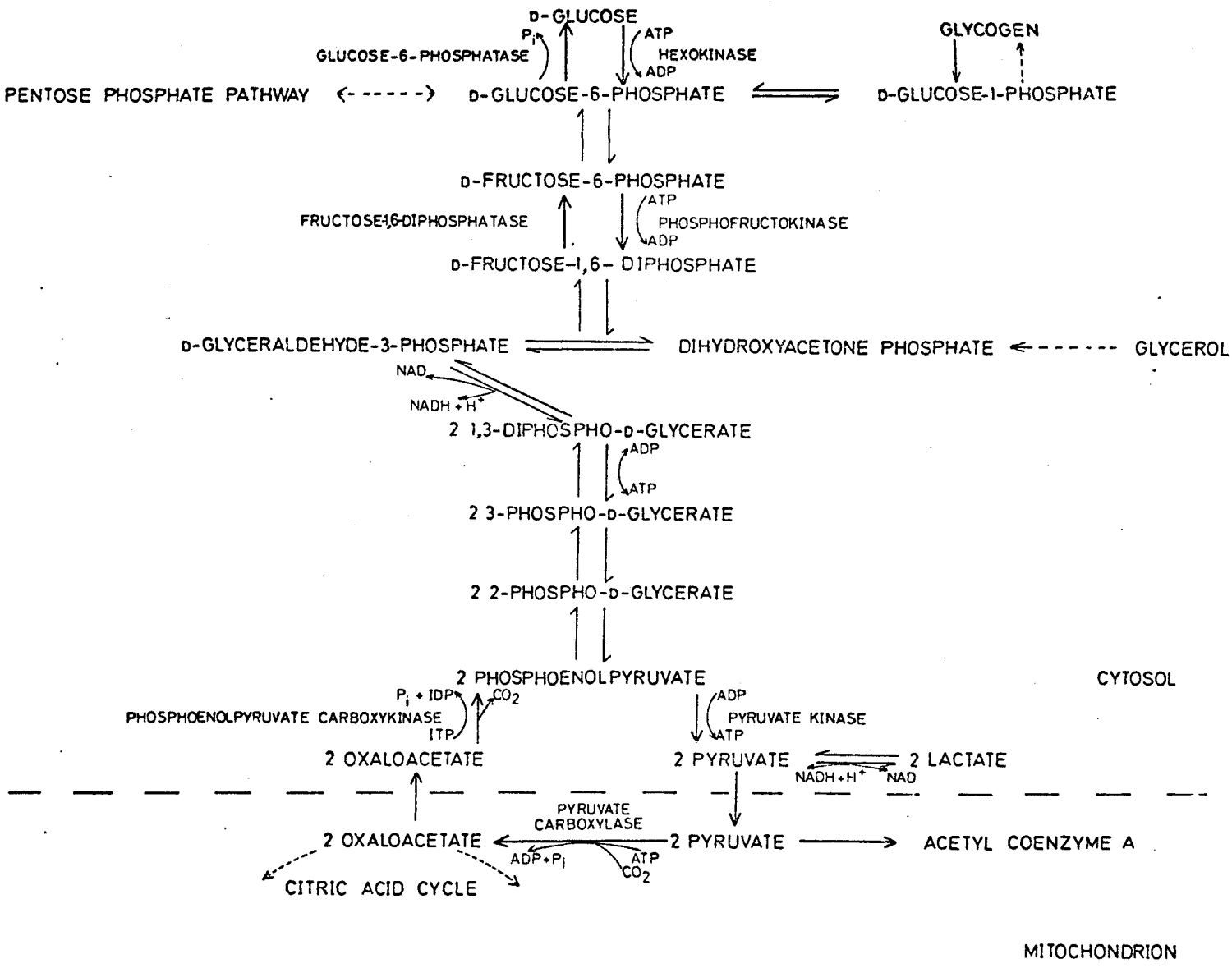


FIGURE 1. The pathways of glycolysis and gluconeogenesis.

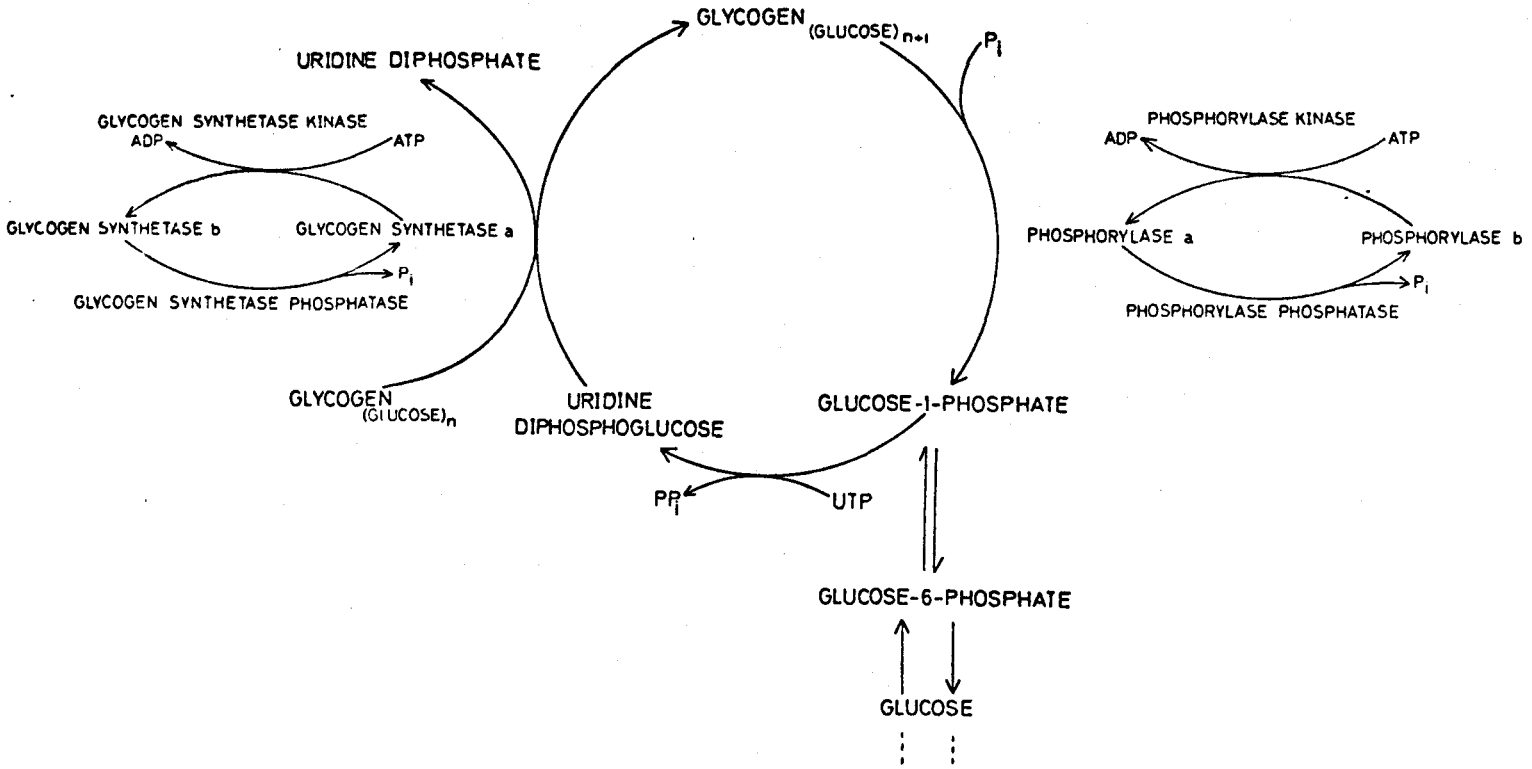


FIGURE 2. The pathways of glycogen synthesis and degradation.

synthesis) and glycogen phosphorylase (glycogen degradation). The activities of these enzymes are regulated by dietary and hormonal factors (Newsholme and Start, 1973).

During the early stages of liver development, the hepatocytes have little or no specialised function and derive energy for cell division from the metabolism of glucose derived from the maternal blood supply. As the cells become more specialised, the activities of key enzymes increase giving the tissue its capacity for glycolysis, gluconeogenesis and glycogen synthesis and degradation. These processes are initiated at different stages of development in different species. In the livers of most species the glycolytic sequence is initiated before birth. In rat there is no capacity for hepatic gluconeogenesis until birth, whereas in other species such as guinea pig and sheep the process is initiated at an earlier stage (Ballard and Oliver, 1963, 1965; Ballard and Hanson, 1967; Arinze, 1975; Jones and Ashton, 1976). Glycogen is not synthesised in foetal liver until a fairly late stage of development in species which have a short gestation period, whereas in species which have a longer gestation period, it is synthesised from early in pregnancy (Dawkins, 1963a, 1963b; Ballard and Oliver, 1963, 1964; Jaquot and Kretchmer, 1964; Gennser *et al.*, 1971; Devos and Hers, 1974).

The early studies of Warburg (1930) indicated differences between the metabolism of normal and tumour tissues. Since the ultimate control of metabolic processes in tissues is dependent on the levels of enzyme activity, it was logical to search for possible changes in tumour enzyme content as the primary cause of the alteration in metabolism. Consequently, a vast literature on the enzymology of many kinds of spontaneous and transplantable tumours now exists.

The production by Morris (Morris, 1963, 1965; Morris and Wagner,

1968) of a series of chemically-induced transplantable rat hepatomas of varying growth rate and differentiation (representative of different degrees of malignancy) enabled ordered studies, aimed at determining the significance of enzyme changes in relation to the severity of the disease, to be carried out.

The studies of Weber and co-workers (Weber and Cantero, 1955a; Weber and Morris, 1963; Weber *et al.*, 1964, 1972; Weber, 1963, 1966, 1972, 1974; Weber and Lea, 1966) have shown that in Morris hepatomas, increasing tumour growth rate and loss of structural differentiation is associated with characteristic but opposing changes in the activities of the key enzymes of the synthetic and catabolic pathways of intermediary metabolism. The activities of key glycolytic enzymes increase in parallel with the decrease in tumour differentiation. The increased rate of glycolysis, as measured by the production of lactate, in the hepatomas corresponds with the increased levels of the glycolytic enzymes. However, the rate of glycolysis in some highly-differentiated 'minimal deviation' hepatomas is in the same range or lower than that of normal liver, refuting the long-held view that all tumours necessarily exhibit an increased rate of glycolysis (Warburg, 1930). In contrast, the activities of the key gluconeogenic enzymes decrease as the hepatomas become less well-differentiated. The changes in enzyme activity correlate closely with the overall reduction in the rate of gluconeogenesis. Moreover, alteration in the normal regulation of gluconeogenic enzymes is observed and the increase in activity which usually follows glucocorticoid administration is reduced in well-differentiated hepatomas and is completely absent in poorly-differentiated tumours. Consistent with these findings are the low levels of glycogen in many hepatomas and the reduced activities of phosphoglucomutase.

Changes in enzyme activities indicative of increased glycolysis,

decreased gluconeogenesis and decreased glycogen synthesis have also been observed by others in Morris, Yoshida, Novikoff and 3'-dimethylaminoazobenzene-induced rat hepatomas (Pitot, 1960; Saheki *et al.*, 1971; Lea *et al.*, 1972; Balinsky *et al.*, 1972; Chang and Morris, 1973; Misheneva *et al.*, 1973; Sato *et al.*, 1973a, 1973b), as well as in human primary hepatomas (Boxer and Shonk, 1966; Balinsky *et al.*, 1973a; Yeung *et al.*, 1973).

Weber considers that the enzyme and metabolic activity of hepatomas constitutes a definitive pattern at the molecular level, which can be explained only in terms of ordered alterations in gene expression. These opinions have been incorporated in the 'molecular correlation concept of neoplasia' (Weber, 1966, 1972, 1974; Weber and Lea, 1966). It is postulated that metabolic homeostasis in tissues is normally maintained by the antagonistic activity of key enzymes regulating the synthetic and catabolic pathways of intermediary metabolism. The key enzymes of each pathway are envisaged as forming separate functional genic units whose co-ordinated activity regulates the overall balance of metabolism. In hepatomas it is proposed that genetically determined alterations in the expression of whole genic units produce changes in the activities of groups of enzymes, with corresponding effects on the balance of metabolism in the pathways concerned.

Regulation of hepatic activity *in vivo* is known to be highly complex. Various cell lines have therefore been developed with a view to studying the regulatory processes *in vitro* in a simple biological system. Studies of cell cultures derived from rat liver and hepatoma by Wicks *et al.* (1973) have shown that the cells do not necessarily retain properties characteristic of the tissue of origin. In Reuber H35 and $M_1H_1C_1$ hepatoma cells, enzyme activities were found to be similar to those of normal liver whereas in HTC hepatoma cells and RLC liver cells the

levels were lower than in normal liver. Reuber H35 and $M_1H_1C_1$ cells were shown to retain certain regulatory features of normal liver whereas HTC and RLC cells did not.

The activities of the key gluconeogenic enzymes, pyruvate carboxylase (pyruvate: carbon dioxide ligase (ADP), E.C.6.4.1.1), phosphoenolpyruvate carboxykinase (GTP: oxaloacetate carboxylase (transphosphorylating), E.C.4.1.1.32) and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C.3.1.3.9) and the key enzyme of glycogen synthesis, glycogen synthetase (UDP glucose: glycogen 4-α-glucosyltransferase, E.C.2.4.1.11) were of interest in this study. These enzymes will therefore be considered in more detail below.

Pyruvate carboxylase

The occurrence of pyruvate carboxylase (PC) was first reported in chicken liver mitochondria by Utter and Keech (1960). The enzyme has subsequently been detected in a variety of vertebrate tissues, although activities in heart, muscle and brain are considerably lower than in liver, kidney cortex or adipose tissue (Utter and Keech, 1963; Ballard *et al.*, 1970; Anderson, 1970).

$$\text{pyruvate} + \text{ATP} + HCO_3^- \xrightarrow[\text{AcCoA}]{Me^{2+}\ Me^{+}} \text{oxaloacetate} + \text{ADP} + P_i$$

Studies carried out on chicken liver have shown that the one-carbon species utilised during the reaction is bicarbonate and not carbon dioxide (Cooper *et al.*, 1968). All pyruvate carboxylases examined so far in vertebrate species, require the presence of an acyl coenzyme A such as acetyl coenzyme A, a divalent metal ion such as magnesium or manganese and a monovalent metal ion such as potassium, rubidium, caesium or sodium, in order to catalyse a significant rate of pyruvate carboxylation (Scrutton

and Young, 1972).

Studies of the subcellular distribution of PC have shown that in rat, guinea pig, rabbit and human livers the enzyme is located predominantly in the mitochondria, with only a small proportion in the cytosol fraction (Ballard and Hanson, 1967; Wieland *et al.*, 1968; Brech *et al.*, 1970; Saggerson and Evans, 1975); this implies that pyruvate is required to enter the mitochondria before being carboxylated to oxaloacetate. However, Henning *et al.* (1963) reported the presence of significant levels of PC activity in the cytosol fraction of rat liver and more recently Dugal (1974) found approximately 40% of the total PC activity in the soluble fractions of rat, pig, bovine and chicken livers. These workers have suggested that pyruvate is not necessarily required to enter the mitochondria prior to its carboxylation.

The total activities of PC have been shown to be similar in livers of adult rat and guinea pig but are somewhat lower in human livers (Wieland *et al.*, 1968; Brech *et al.*, 1970; Söling *et al.*, 1970).

Changes in the activity of PC in rat liver have been noted in response to dietary and hormonal changes. The activity of the enzyme increases on starvation (Ashmore *et al.*, 1964; Freedman and Kohn, 1964; Wimhurst and Manchester, 1973) and in response to glucocorticoids (Henning *et al.*, 1963; Prinz and Seubert, 1964; Ashmore *et al.*, 1964). Henning *et al.* (1963), Prinz and Seubert (1964) and Freedman and Kohn (1964) found PC activity to be increased in the livers of diabetic rats whereas Krebs (1966), Shrago and Lardy (1966) and Brech *et al.* (1970) did not observe such an increase.

Ballard and Hanson (1967) reported that PC activity occurs in the mitochondrial fraction of foetal rat livers. The activities of PC in

livers of rat foetuses of 17 - 19 days gestational age are very low and increase gradually to term (21 days), reaching adult levels shortly after birth (Ballard and Hanson, 1967; Chang and Morris, 1973). In foetal guinea pig liver, PC is almost exclusively mitochondrial and at about 50 days (the gestation period is approximately 70 days) there is significant activity, which rises to values several times higher than those in adult liver and then falls after birth (Jones and Ashton, 1976). PC activity has been demonstrated in foetal sheep livers from as early as 47 days gestational age (the gestation period is 147 days) and has been shown to rise gradually during development until adult levels are reached shortly after birth (Warnes *et al.*, 1974; Edwards *et al.*, 1975). Significant levels of PC have also been reported in human foetal livers from an early stage of gestation (8 -12 weeks) (Räihä and Lindros, 1969).

The activities of PC in a series of rat host livers from animals carrying experimental hepatomas of different growth rates have been shown to be similar to or slightly lower than the levels in normal livers (Chang and Morris, 1973).

In rat hepatomas, the total activities of PC have been found to be lower than the levels in normal or host livers and to decrease with increasing tumour growth rate and loss of differentiation (Weber, 1963, 1966, 1974). However, studies of mitochondrial PC in Morris hepatomas have shown that although the levels are reduced in most cases, there does not appear to be a good correlation between enzyme activity and growth rate or degree of differentiation (Chang and Morris, 1973). Enzymatic, electrophoretic and immunological studies showed the properties of rat hepatoma PC to be similar to those of the enzyme from the livers of adult and new-born rats (Chang and Morris, 1973). The levels of PC in human hepatomas have also been found to be lower than those in normal livers, as demonstrated by the

work of Yeung *et al.* (1973) on non-hypoglycaemic cancer patients in China.

Phosphoenolpyruvate carboxykinase

Phosphoenolpyruvate carboxykinase (PEPCK) was first observed in chicken liver by Utter and Kurahashi (1954a). The enzyme has since been shown to occur in tissues of a wide variety of species (Utter and Kolenbrander, 1972). Relatively high activities are found in the gluconeogenic tissues, liver and kidney. Lower levels are found in other tissues including mammary, certain types of muscle and adipose tissue.

$$\text{phosphoenolpyruvate} + CO_2 + \text{NDP} \overset{Me^{2+}}{\rightleftharpoons} \text{oxaloacetate} + \text{NTP}$$

The enzyme catalyses the reversible reaction shown above, the physiological direction being the formation of oxaloacetate. NDP and NTP represent nucleoside di- and triphosphates, respectively. In vertebrate species, the enzyme is specific for inosine or guanosine phosphates and in addition a divalent metal ion, usually manganese or magnesium, is required (Utter and Kolenbrander, 1972). Studies of bacterial PEPCK have shown that the one-carbon species utilised in the carboxylation direction is carbon dioxide and not the bicarbonate ion (Cooper *et al.*, 1968).

The subcellular distribution of PEPCK varies from one species to another. In rat, mouse and hamster livers the enzyme is found mainly in the cytosol (Nordlie and Lardy, 1963; Ballard and Hanson, 1967) whereas in rabbit, chicken and pigeon livers it is essentially mitochondrial (Utter, 1959; Nordlie and Lardy, 1963; Felicioli *et al.*, 1967; Gevers, 1967; Johnson *et al.*, 1973). PEPCK is almost equally distributed between the cytosol and mitochondrial fractions of guinea pig, pig, cat, sheep, bovine and human livers (Nordlie and Lardy, 1963; Ballard *et al.*, 1968; Wieland *et al.*, 1968; Brech *et al.*, 1970; Söling *et al.*, 1970; Swiatek *et al.*, 1970; Garber and Hanson, 1971a; Smith and Osborne-White, 1971; Hanson

and Garber, 1972).

It has been suggested that in rat liver, oxaloacetate formed in the mitochondria during the PC reaction is converted to malate or aspartate for transport across the mitochondrial membrane and subsequently regenerated from these intermediates to form phosphoenolpyruvate in the cytosol (Lardy *et al.*, 1965; Krebs *et al.*, 1967). However, Henning *et al.* (1963) and Dugal (1974) who, unlike other workers, found significant amounts of PC in the soluble fraction of rat liver, have suggested that phosphoenolpyruvate can be formed directly in the cytosol from oxaloacetate. The situation in species in which a significant proportion of hepatic PEPCK activity is present in the mitochondrial fraction is presumably different from that in rat; it has been demonstrated that in guinea pig and rabbit livers, both the cytosol and mitochondrial forms of PEPCK actively function during gluconeogenesis (Arinze *et al.*, 1973).

Similar values have been reported for the total activities of PEPCK in rat and human livers (Brech *et al.*, 1970) whereas the levels in guinea pig livers have been found to be somewhat higher than those in rat livers (Soling *et al.*, 1970, 1973).

Hepatic PEPCK activity is increased on starvation and in diabetes (Shrago *et al.*, 1963; Foster *et al.*, 1966a; Pogson and Smith, 1975) and by administration of glucagon or dibutyryl cyclic AMP (Shrago *et al.*, 1963; Wicks *et al.*, 1969; Reshef and Hanson, 1972). Such changes are due to an increase in the rate of synthesis of the enzyme (Wicks *et al.*, 1972; Hopgood *et al.*, 1973; Hanson *et al.*, 1973; Tilghman *et al.*, 1974). Insulin treatment of diabetic rats produces a decrease in enzyme activity (Shrago *et al.*, 1963; Foster *et al.*, 1966a). Shrago *et al.* (1963), Ray *et al.* (1964), Foster *et al.* (1966a) and Huttner *et al.* (1974) have reported that injection of glucocorticoids into intact rats

causes an elevation in hepatic PEPCK activity, whereas Reshef *et al.* (1969, 1970) and Gunn *et al.* (1975) noted such an increase only in diabetic animals. Exton *et al.* (1973) reported that in diabetic adrenalectomised rats the enzyme was induced by glucocorticoids. Gunn *et al.* (1975) have suggested that glucocorticoids increase hepatic PEPCK activity only in the absence of insulin and that the injection of glucocorticoids into intact animals results in insulin secretion, thereby actually decreasing the rate of synthesis of the enzyme.

In the livers of most species it appears that only the cytosol form of PEPCK is adaptive to dietary and hormonal changes, the activity of the mitochondrial enzyme remaining unchanged (Nordlie *et al.*, 1965b; Gevers, 1967; Söling *et al.*, 1970; Garber and Hanson, 1971a, 1971b; Johnson *et al.*, 1970, 1973; Hanson, 1974; Huibregtse *et al.*, 1976). Increases in mitochondrial PEPCK activity have, however, been noted in chicken and pig livers following starvation (Swiatek *et al.*, 1970; Jo *et al.*, 1974).

The mitochondrial formation of phosphoenolpyruvate is of considerable importance in species in which there is a significant amount of PEPCK in this fraction and is markedly decreased by fatty acids and by β-hydroxybutyrate, which act by increasing mitochondrial NADH levels (Garber and Hanson, 1971a, 1971b; Jomain-Baum and Hanson, 1973; Arinze and Hanson, 1973; Arinze *et al.*, 1973). These compounds inhibit gluconeogenesis in isolated, perfused livers of guinea pig and rabbit but stimulate the process in rat liver (Söling *et al.*, 1970; Arinze *et al.*, 1973). Since shifting the mitochondrial oxidation-reduction potential towards increased reduction inhibits gluconeogenesis, Garber and Hanson (1971a, 1971b), Jomain-Baum and Hanson (1973) and Arinze and Hanson (1973) have advanced the hypothesis that the oxidation-reduction state is a major factor regulating the mitochondrial formation of phosphoenolpyruvate.

In foetal rat livers, PEPCK occurs mainly in the mitochondria and the activity is low. At birth cytosol PEPCK appears and the activity increases very rapidly within the next forty-eight hours (Ballard and Hanson, 1967). There is no capacity for gluconeogenesis in foetal rat livers before birth (Ballard and Oliver, 1963, 1965; Yeung and Oliver, 1967; Philippidis and Ballard, 1969) and it has been suggested that the appearance of the cytosol enzyme initiates the gluconeogenic sequence (Ballard and Hanson, 1967). Other species such as the guinea pig and sheep, however, have a capacity for gluconeogenesis before birth (Ballard and Oliver, 1965; Arinze, 1975; Jones and Ashton, 1976). PEPCK appears in foetal guinea pig livers from day 30 onwards and is predominantly mitochondrial; at birth the activity of the mitochondrial enzyme decreases while that of the cytosol form increases (Jones and Ashton, 1976). In foetal sheep livers PEPCK occurs in both the mitochondrial and cytosol fractions with a slightly greater proportion in the mitochondria (Warnes *et al.*, 1974). There is significant activity from 47 days onwards, increasing gradually during development (Edwards *et al.*, 1975). Rãihã and Lindros (1969) have reported the presence of low levels of PEPCK in human foetal livers.

The activity of PEPCK in experimental rat hepatomas is lower than in normal livers and decreases with increasing tumour growth rate and loss of differentiation (Weber, 1963, 1966, 1974). In human hepatomas PEPCK activity is lower than in normal liver (Yeung *et al.*, 1973).

The levels of PEPCK in Reuber H35 hepatoma cells (derived from a minimal deviation hepatoma) and $M_1H_1C_1$ hepatoma cells are similar to those of normal rat liver whereas the levels in HTC hepatoma cells (derived from Morris hepatoma 7288-C) and RLC rat liver cells are lower than in normal liver (Wicks *et al.*, 1973). Reuber H35 and $M_1H_1C_1$ cells have been shown to retain certain regulatory features of normal liver in that PEPCK activity is increased in the presence of dexamethazone,

dibutyryl cyclic AMP and other cyclic AMP analogues (Barnett and Wicks, 1971; Wicks and McKibbin, 1972; Wicks *et al.*, 1973; Wagner *et al.*, 1975). Insulin antagonises the stimulatory effects of these substances. The activity of PEPCK in HTC and RLC cells is not affected by the presence of dexamethazone or dibutyryl cyclic AMP in the growth medium (Wicks *et al.*, 1973).

Glucose-6-phosphatase

Glucose-6-phosphatase (G6Pase) was first observed in rabbit liver extracts (Fantl and Rome, 1945) and has subsequently been found to occur in a wide variety of tissues and organs from a large number of species (Nordlie, 1971).

$$\text{glucose-6-phosphate} + H_2O \longrightarrow \text{glucose} + P_i$$

In the liver, kidney, pancreas and intestine of all species examined, the enzyme is localised mainly in the microsomal fraction and is part of, or is strongly attached to, the lipoprotein membrane of the endoplasmic reticulum (Ernster *et al.*, 1962; Stetten, 1964; Lygre and Nordlie, 1968; Nordlie, 1971). A relationship between G6Pase and microsomal phospholipid has been established, suggesting that one of the functions of membrane lipids is to stabilise the conformation of the enzyme which is required for activity (Beaufay and De Duve, 1954; Nordlie, 1971). As might be expected on the basis of the relationship between G6Pase and phospholipids, enzyme activity is influenced by a variety of detergents (Beaufay and De Duve, 1954; Segal and Washko, 1959; Carruthers and Baumler, 1962; Snoke and Nordlie, 1967; Nordlie and Snoke, 1967).

In adult tissues, G6Pase activity is highly responsive to

a wide variety of hormonal and dietary factors. Increases in activity have been noted in response to fasting, high protein, high fat, high galactose and high fructose diets, diabetes, glucagon and glucocorticoids (Ashmore and Weber, 1955; Segal and Washko, 1959; Arion and Nordlie, 1965, 1967; Nordlie *et al.*, 1965a, 1968; Fisher and Stetten, 1966; Pandhi and Baum, 1967; Stetten and Burnett, 1967; Collip, 1967; Snoke and Nordlie, 1967; Nordlie and Snoke, 1967; Nordlie, 1971). Insulin administration reduces the activity in both normal and diabetic animals (Ashmore and Weber, 1955).

As is also the case for PC and PEPCK, the activty of G6Pase in foetal rat livers is low whereas in foetal guinea pig and sheep livers significant levels of the enzyme are present before birth. In rat, hepatic G6Pase activity increases markedly at term, reaching levels 2 - 3-fold higher than adult levels a few days after birth (Dawkins, 1961; Weber and Cantero, 1955b; Burch *et al.*, 1963; Greengard, 1969a, 1969b). G6Pase appears in foetal guinea pig livers at approximately 40 days and the activity increases as much as 10-fold just after birth (Jones and Ashton, 1976). The activity of the enzyme in foetal sheep livers increases only slightly at birth (Warnes *et al.*, 1974). Aurrichio and Rigillo (1960), Gennser *et al.* (1971) and Schwartz *et al.* (1974) have reported the presence of G6Pase in human foetal livers from an early stage of gestation. These workers found that the levels were lower than adult levels and that there did not appear to be a correlation between enzyme activity and foetal age.

The levels of G6Pase have been shown to be similar in normal rat livers and in the livers of rats bearing Novikoff hepatomas (Weber

and Cantero, 1955a).

In experimental rat hepatomas, G6Pase activity has been shown to be considerably reduced with respect to normal or host liver values and, as for PC and PEPCK, there is a tendency for the levels to decrease with increasing growth rate of the tumour (Weber and Cantero, 1955a; Pitot, 1960; Weber and Morris, 1963; Sugimura *et al.*, 1966; Weber, 1963, 1966, 1974). Reduced levels of G6Pase have also been reported in human hepatomas (Yeung *et al.*, 1973).

The levels of G6Pase in cell lines or suspensions established from spontaneous mouse mammary carcinomas and uterine sarcomas and from thioacetamide-induced mouse hepatomas have been reported to be similar to or lower than the levels in the corresponding tumour tissues (Bhide, 1970; Wilson, 1973).

Glycogen synthetase

Glycogen synthetase (GS), which catalyses the synthesis of glycogen from uridine diphosphoglucose, is widely distributed in tissues of a large number of species, the activities being highest in liver, muscle and heart (Knox, 1972; Stalmans and Hers, 1973).

$$\text{UDPG} + \text{glycogen}_{(\text{glucose})_n} \longrightarrow \text{UDP} + \text{glycogen}_{(\text{glucose})_{n+1}}$$

Differential centrifugation studies of mammalian tissues have shown that most of the GS activity is recovered with particulate glycogen and that the amount of enzyme in other fractions is proportional to their glycogen content (Robbins *et al.*, 1959; Leloir and Goldemberg, 1960).

Villar-Palasi and Larner (1960) first recognised the existence of two forms of GS which are interconvertible through phosphorylation

by a kinase and dephosphorylation by a phosphatase. The dephosphorylated form, GS a, is active in the absence of glucose-6-phosphate, whereas the phosphorylated form, GS b, requires glucose-6-phosphate for activity. Subsequent studies have shown that in rat tissues, GS a is only slightly inhibited by ADP, ATP and phosphate ions and that the inhibition is independent of the glucose-6-phosphate concentration. GS b, however, is strongly inhibited by these compounds and glucose-6-phosphate relieves the inhibition but only when present at high concentrations (Gold and Segal, 1967; Piras *et al.*, 1968; Hers *et al.*, 1970). Under physiological conditions, therefore, GS b is almost totally inactive and is not modified by glucose-6-phosphate.

The relative activities of GS a and GS b in normal adult livers are dependent on the activities of the interconverting enzymes and are subject to dietary and hormonal control (Newsholme and Start, 1973; Stalmans and Hers, 1973). Increase in blood glucose concentration resulting from food intake causes deposition of glycogen in the liver which is entirely explained by the activation of GS (De Wulf and Hers, 1967a). It appears that this effect is mediated by the inactivation of phosphorylase a which is an inhibitor of synthetase phosphatase (Stalmans and Hers, 1973). As glycogen accumulates in the liver, a feed-back mechanism is operative whereby synthetase phosphatase activity is inhibited and the GS a to GS b conversion is facilitated, resulting in a decrease in glycogen synthesis (Hers *et al.*, 1970; Newsholme and Start, 1973). Hepatic GS is activated following insulin administration and it is probable that the effect, like that of glucose, may be secondary to inactivation of phosphorylase (Bishop *et al.*, 1971). Administration of glucagon, epinephrine or cyclic AMP causes a rapid inactivation of liver GS (Bishop and Larner, 1967; De Wulf and Hers, 1968). It appears that the effect of glucagon is mediated by the

formation of cyclic AMP; activation of protein kinase by the cyclic nucleotide causes the conversion of GS a to GS b and simultaneously the activation of phosphorylase kinase and formation of phosphorylase a (Stalmans and Hers, 1973). Administration of glucocorticoids induces deposition of glycogen in the liver associated with increased GS a activity and decreased phosphorylase a activity (Hornbrook *et al.*, 1966; De Wulf and Hers, 1967b; Kreutner and Goldberg, 1967).

In species such as the rat, rabbit and guinea pig, which have a short gestation period, glycogen is virtually absent from the liver until the last fifth of pregnancy (Jost, 1960; Shelley, 1961; Kornfeld and Brown, 1962; Ballard and Oliver, 1963; Dawkins, 1963b; Jaquot and Kretchmer, 1964; Burch, 1965; Barnes, 1968; Devos and Hers, 1974). In the livers of species such as sheep, monkey and human, which have a longer gestation period, glycogen is present as early as the second quarter of pregnancy (Villee, 1953; Shelley, 1961; Dawkins, 1963a; Ballard and Oliver, 1964; Gennser *et al.*, 1971). Rapid increases in hepatic glycogen levels just before birth have been observed in all mammalian species studied so far (Barnes, 1968; Gennser *et al.*, 1971; Devos and Hers, 1974). In foetal rat liver, the appearance of glycogen is paralleled by the appearance of GS a, although GS b may be present at an earlier stage. GS a activity increases as glycogen accumulates and then drops just before term when glycogen levels are high (Jaquot and Kretchmer, 1964; Burch, 1965; Schwartz and Rall, 1973; Devos and Hers, 1974). Accumulation of glycogen in foetal rat liver has been shown to be due to a relatively slow rate of synthesis and a virtual absence of degradation (Devos and Hers, 1974).

Studies carried out on experimental rat hepatomas have shown that glycogen levels and GS activities (both a and b forms) are in most cases lower in the tumours than in normal livers and that, in general, glycogen

metabolism in fast-growing, poorly-differentiated hepatomas differs to a greater extent from that in normal livers than does glycogen metabolism in slow-growing, well-differentiated tumours (Nigam, 1967; Saheki *et al.*, 1971; Lea *et al.*, 1972; Misheneva *et al.*, 1973; Sato *et al.*, 1973a, 1973b). In faster-growing hepatomas, the properties of GS have been shown to differ from those of the liver enzyme and to resemble those of muscle GS (Sato *et al.*, 1972a, 1973a, 1973b).

Alpers *et al.* (1963) have shown that glycogen is deposited and GS activity increased in HeLa cells grown in the presence of high concentrations of glucose. Bernaert *et al.* (1974) showed the formation of glycogen particles in cultured rat hepatocytes to be associated with conversion of GS b to GS a initially and then reconversion of GS a to GS b as glycogen accumulates. A high proportion of GS b has been reported in Ehrlich ascites carcinoma cells, which have been shown to contain an 'endogenous factor' which inhibits the conversion of GS b to GS a (Baba and Tsuiki, 1974).

Isozymes in normal adult and foetal tissues, tumour tissues and cell cultures

General

For some time it has been recognised that many enzymes can exist in multiple molecular forms or isozymes, which may be distinguished by means of their physical, chemical and immunological properties. The differences between the properties of the isozymes of a particular enzyme frequently appear to be of functional significance. In addition to determining total levels of enzyme activity, it is therefore important to determine the proportions of the various isozymes present in different

tissues. This is usually achieved by means of electrophoretic, isoelectric focusing or chromatographic procedures.

The enzymes of carbohydrate metabolism which are well known to exist in multiple forms include phosphoglucomutase, glucose-6-phosphate dehydrogenase, hexokinase, phosphofructokinase, aldolase, pyruvate kinase, lactate dehydrogenase and fructose-1,6-diphosphatase. These enzymes have been studied extensively and a considerable literature concerning their isozyme patterns in a wide variety of tissues now exists. In some cases (e.g. phosphoglucomutase and glucose-6-phosphate dehydrogenase) isozyme patterns in the same tissue differ from one individual to another as a result of genetic variation. Tissue-specific differences in isozyme distributions (associated with functional differences) and species-specific differences in isozyme mobilities are frequently observed.

During the foetal and neonatal stages of development as the cells become more specialised, the isozyme patterns characteristic of adult tissues are acquired. The appearance of each of the different isozymes occurs at a characteristic stage of development in a particular species (Pfleiderer and Wachsmuth, 1961; Fine *et al.*, 1963; Benson and de Jong, 1968; Sigman *et al.*, 1972; Osterman *et al.*, 1973).

In experimental rat hepatomas, isozymes which play a functional role in liver metabolism are often replaced by isozymes which are low or absent in the adult differentiated tissue (Criss, 1971; Schapira, 1973). Some of the principal changes in the isozyme patterns of enzymes of carbohydrate metabolism in rat hepatomas are summarised in Table I. The isozymes present in hepatoma are often forms characteristic of other adult tissues or of undifferentiated foetal or neonatal liver.

Loss of liver-specific isozymes and their replacement by other forms has also been observed in human hepatomas (Balinsky *et al.*, 1973a).

TABLE I

ISOZYME CHANGES IN RAT HEPATOMAS

ENZYME	ISOZYMES IN NORMAL TISSUES		ISOZYME CHANGES IN HEPATOMA
Hexokinase	Liver:	I	
		II	Increased II
		III	Decreased III
		IV	Decreased IV
	Muscle:	I	
		II	
	Foetal liver: (early stage)	I	
		IA	
		II	
Aldolase	Liver:	B	
	Muscle	A	Increased A
	Brain:	C	
Pyruvate kinase	Liver:	L (major form)	Decreased L
		M_2 (minor form)	Increased M_2
	Foetal liver:	M_2 (major form)	
		L (minor form)	
	Muscle:	M_1	
Lactate dehydrogenase	Liver:	Mainly M subunits	Slight increase in H subunits in some cases
	Foetal liver:	Slightly greater proportion of H subunits than in adult liver	
	Muscle:	Mainly M subunits	
	Heart:	Mainly H subunits	
Fructose-1,6-diphosphatase	Liver:	L	Decreased L
	Muscle:	M	Increased M

The information given in this Table has been taken from the reviews of Criss (1971) and Schapira (1973).

Isozyme patterns in a wide variety of other tumour tissues of various species, including rat and human have been shown to differ from those in the corresponding normal tissues (Criss, 1971; Langvad, 1972; Kikuchi *et al.*, 1972; Sato *et al.*, 1972c; Schapira, 1973; Spellman and Fottrell, 1973).

The trend towards the foetal pattern in rat hepatomas, led Schapira (1966) to suggest that in cancer, synthesis of tissue specific adult isozymes is repressed whereas that of embryonic forms is derepressed. This concept has been amplified by Potter (Potter, 1969; Walker and Potter, 1972) in what he has termed 'oncogeny as blocked ontogeny'. It was observed that the potential for synthesis of certain foetal isozymes is retained in adult rat liver and when fully mature hepatocytes are stimulated to undergo cell division they cease production of some of the adult isozymes and revert to production of foetal forms. This process 're-ontogeny' was related to the processes of cell division irrespective of the initiating cause. The same degree of differentiation was observed in regenerating liver as in the nodular precancerous liver following carcinogen administration. However, in the regenerating liver, it appears that the process is controlled and the newly formed hepatocytes undergo differentiation to generate fully mature liver cells with a normal complement of adult isozymes. Similarly in the precancerous liver it is probable that many hyperplastic cells may undergo complete differentiation but the 'blocked ontogeny' hypothesis postulates that some cells are unable to complete this process and develop into clones of hepatoma cells. Hepatomas of diverse enzymic phenotype would thus be produced depending upon the combination of blocks and the stages at which blockages of dedifferentiation occur. Matsushima *et al.* (1968) expressed this concept as a "disdifferentiation" and more generally Markert (1968) postulates that cancer is a disease of differentiation.

In cell cultures the isozymes of a given species retain their characteristic mobilities, but the proportions of the various forms may not be characteristic of the tissue of origin; in general, rapidly-dividing cells originating from different tissues of the same species show a uniform pattern (Nitowsky and Soderman, 1964; Vesell, 1965; Katzen *et al.*, 1965; Montes de Oca *et al.*, 1969; Walker *et al.*, 1972). Complex structural and biochemical interactions are not maintained in culture and changes in isozyme patterns presumably result from adaptation of the cells to the culture conditions. Walker *et al.* (1972) suggested that stimulation of cell division during culture may cause the cells to lose proteins characteristic of the adult state and change to synthesis of proteins characteristic of the early stages of development. It is also possible that in primary cultures selection occurs for a cell containing isozymes which would give it an advantage for cell division.

In this work the isozyme patterns of phosphoglucomutase (α-D-glucose-1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase, E.C.2.7.5.1), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$1-oxidoreductase, E.C.1.1.1.49), hexokinase (ATP:D-hexose 6-phosphotransferase, E.C.2.7.1.1), pyruvate kinase (ATP:pyruvate 2-0-phosphotransferase, E.C.2.7.1.40) and lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, E.C.1.1.1.49) were of interest. These enzymes will therefore be considered in detail below.

Phosphoglucomutase

Phosphoglucomutase (PGM), which catalyses the reversible transfer of phosphate between the 1 and 6 positions of glucose, has been observed in a wide variety of avian, amphibian and mammalian tissues (Spencer *et al.*, 1964; Dawson and Jaeger, 1970; Dawson and Greene, 1975).

glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate

Multiple forms of the enzyme (usually four or five in mammals) have been separated by means of ion-exchange chromatography, electrophoresis and isoelectric focusing (Harshman and Six, 1969; Dawson and Jaeger, 1970; Greene and Dawson, 1973; Dawson and Greene, 1975). On starch gel electrophoresis the intensity of the bands decreases as the mobility towards the anode increases and it appears, from studies carried out on rabbit, that the different isozymes probably exist *in vivo* and are caused by progressive oxidation of free sulphydryl groups (Greene and Dawson, 1973; Dawson and Greene, 1975).

In human subjects, PGM shows genetic variation and electrophoretic patterns corresponding to approximately twenty different phenotypes have been described, the common variants being PGM_1, PGM_{2-1} and PGM_2 (Spencer *et al.*, 1964; Hopkinson and Harris, 1965, 1966). The PGM isozyme patterns have been shown to be identical in normal tissue extracts derived from the same individual (Spencer *et al.*, 1964).

The activity of PGM in rat hepatomas has been shown to be lower than in normal or host livers (Weber *et al.*, 1964; Balinsky *et al.*, 1972). Weber *et al.* (1964) found the Michaelis constant of PGM for glucose-1-phosphate to be higher in two fast-growing rat hepatomas than in normal liver but obtained a value of this constant similar to that of normal liver for a slow-growing hepatoma.

In cell cultures derived from various human tissues, the PGM isozyme patterns were found to correspond to those of the tissue of origin (Spencer *et al.*, 1964).

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) directs glucose-6-phosphate into the pentose phosphate pathway by catalysing its conversion to

6-phosphogluconolactone.

$$\text{glucose-6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconolactone} + NADPH + H^+$$

Watanabe and Taketa (1971) and Taketa and Watanabe (1971) showed that, by means of polyacrylamide gel and cellulose acetate electrophoresis, rat liver G6PD can be separated into three major components (designated types I, II and III, in order of increasing mobility towards the anode) which run closely together and appear almost like sub-bands. Only the two slower components are found in freshly-prepared liver homogenates from well-fed rats, whereas all three bands are detected in livers from starved rats which have been re-fed. A purified rat liver G6PD preparation has been shown to have three or four major components as well as three minor slower-moving bands (Holten, 1972). The major forms of G6PD in rat liver are interconvertible; in aged extracts the proportions of the faster major components increase and in addition minor bands of varying number and intensity are sometimes detected (Watanabe and Taketa, 1971; Taketa and Watanabe, 1971; Holten, 1972).

Human G6PD shows genetic variation and a large number of variants, many of which have differing electrophoretic mobilities, have been reported (Kirkman, 1971; Beutler, 1972). The normal form is the B variant which is the only form found in Caucasians, except for rare variants. Some Negroes possess an enzyme with faster than normal electrophoretic mobility, the A variant (Boyer *et al.*, 1962). The A variant can be either A^+ corresponding to normal enzyme activity or A^- which corresponds to deficient enzyme activity. Most other G6PD variants occur in enzyme-deficient individuals and are in some cases associated with haemolytic disorders (Kirkman, 1971; Beutler, 1972).

The starch and polyacrylamide gel electrophoretic patterns of human erythrocyte G6PD have been studied extensively (Kirkman and Hanna,

1968; Kirkman, 1971). In normal individuals, a single major band corresponding to either the A or B phenotype is observed. In some instances, fine subcomponents have been distinguished in the major bands and faint slower-moving bands have also been detected. When electrophoresis was carried out using a discontinuous borate buffer system, a fast-moving band was found to be present and it has been suggested that this represents the monomeric form of the enzyme, the usual major bands representing the dimeric form. In extracts prepared from human liver, two faint slow-moving bands have been detected in addition to the major forms (Kirkman and Hanna, 1968).

Comparison of G6PD isozyme patterns in rat liver and hepatoma have shown that in addition to the two major components present in normal and host liver (types I and II), hepatoma AH 30 has the faster sub-band (type III) (Taketa and Watanabe, 1971).

Hexokinase

The key glycolytic enzyme, hexokinase (HK), catalyses the phosphorylation of glucose to form glucose-6-phosphate.

$$\text{glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}$$

The first evidence that more than one type of hexokinase existed in mammalian cells was the discovery of Dipietro *et al.* (1962) that liver contained a "glucokinase", which required a very high concentration of glucose for saturation and thus appeared to account for the requirement of high blood glucose concentrations for glycogen synthesis in the liver. The subsequent work of González *et al.* (1964) and Katzen *et al.* (1965) demonstrated the presence in rat liver of four distinct HK isozymes, separable by means of ion-exchange chromatography and starch gel electrophoresis. These isozymes, which also occur in other species and are present in

different proportions in different tissues, have been designated HK I, HK II, HK III and HK IV, in order of increasing electrophoretic mobility towards the anode (Katzen *et al.*, 1965; Grossbard *et al.* 1966). HK I, HK II and HK III can be detected at low glucose concentrations whereas HK IV (glucokinase) requires a high concentration of glucose (Katzen *et al.*, 1965).

The starch gel electrophoretic HK isozyme patterns in normal adult rat tissues have been well studied (Katzen *et al.*, 1965; Katzen and Schimke, 1965; Grossbard *et al.*, 1966). The predominant isozyme in muscle is HK II and in addition HK I is present. All four isozymes occur in liver and HK I is the major form. Brain contains mainly HK I and kidney contains HK I and a trace of HK III. In fat pad and heart there are almost equal amounts of HK I and HK II.

Under certain conditions splitting of HK II and HK IV has been observed in rat tissues. Tryptic hydrolysis of HK II gives rise to an isozyme intermediate between HK II and HK III; a similar effect is observed in the presence of glucose (Grossbard and Schimke, 1966). When electrophoresis was carried out in the presence of EDTA but without mercaptoethanol in the buffers, Katzen (1966) reported splitting of HK II of adipose tissue and muscle into a slower band (II_b) and a faster band (II_a). Shatton *et al.* (1969) have, however, occasionally observed splitting of HK II even in the presence of mercaptoethanol. Pilkis and Hansen (1968) have reported splitting of HK IV into two components, a fast-moving form (IV_f) and a slow moving form (IV_s) but claim that this only occurs when electrophoresis is performed in the absence of EDTA, the presence or absence of mercaptoethanol being without effect. Sato *et al.* (1969) have, however, reported that splitting of HK IV occurs even in the presence of EDTA in the electrophoresis buffers. Splitting of HK IV in the absence of both EDTA and mercaptoethanol has been reported by Sigman *et al.* (1972).

The studies of Sapag-Hagar *et al.* (1969) have shown that activity due to HK I, HK II and HK III is associated mainly with the non-parenchymal tissue of liver, whereas HK IV is restricted to the hepatocytes. A considerable proportion of the HK activity in tissue homogenates is associated with the mitochondria. Katzen *et al.* (1970) have shown that the hexokinases found in the mitochondria are indistinguishable from HK I and HK II found in the cytosol and that HK III does not occur in particulate form.

The isozymes HK II_a and HK IV of rat tissues are subject to nutritional and hormonal control. The proportions of HK II_a in fat pad, muscle and heart and HK IV in liver decrease during starvation and in diabetes and increase as a result of refeeding or insulin administration (Sharma *et al.*, 1963; Niemeyer *et al.*, 1963; Katzen and Schimke, 1965; Katzen, 1966).

The kinetic properties of hexokinase isozymes isolated from rat tissues have been studied by Grossbard and Schimke (1966) and González *et al.* (1967). The Michaelis constants of HK IV for glucose, 2-deoxyglucose and fructose are considerably higher than for the other three isozymes. The Michaelis constants for glucose are, in terms of order of magnitude, 10^{-5}, 10^{-4}, 10^{-6} and 10^{-2}M, for HK I, HK II, HK III and HK IV, respectively. HK III is inhibited at high glucose concentrations whereas the other isozymes are unaffected. HK I, HK II and HK IV are inhibited to a greater extent by glucose-6-phosphate than HK III. HK I and HK III are inhibited to a greater extent by ADP than HK II.

The HK isozyme patterns in human tissues are somewhat more variable than in rat and differences between the respective tissue distributions are apparent. Neumann *et al.* (1974) have reported that in most cases only HK I is present in human muscle obtained at autopsy,

whereas Benson and de Jong (1968) and Rogers *et al.* (1975) have detected a small proportion of HK II in addition to HK I in this tissue. Balinsky *et al.* (1973a), Neumann *et al.* (1974) and Rogers *et al.* (1975) found human liver obtained at autopsy to have HK I and HK III. However, Benson and de Jong (1968) using autopsy material and Brown *et al.* (1967) using surgical material reported the presence of all four HK isozymes in liver. Brown *et al.* (1967) found the proportion of HK IV to be reduced in livers of poorly-nourished individuals. Brain, kidney, fat pad and heart obtained at autopsy have been found to have only HK I (Neumann *et al.*, 1974). Splitting of human hexokinases I, II and III has been observed by Rogers *et al.* (1975), who did not include DTT or mercaptoethanol in the electrophoresis buffers.

Kinetic studies of human hexokinases have been carried out by Neumann *et al.* (1974) (HK I and HK III) and Cayanis and Balinsky (1975) (HK I, HK II and HK III). As is the case in rat, the Michaelis constants of the human isozymes for glucose increase in the order HK III < HK I < HK II and the only isozyme which is inhibited by high concentrations of glucose is HK III. All three human isozymes have similar Michaelis constants for ATP.

The foetal development of rat liver HK isozymes has been studied by Sigman *et al.* (1972). HK I and HK II are present from as early as 13 days gestation and between days 15 and 16, HK III can also be identified. HK IV appears at approximately 18 days and it is possible that synthesis of insulin might be the stimulus for its induction. An additional isozyme (HK IA), of mobility intermediate between HK I and HK II, is present in the livers of foetuses ranging from 14 - 21 days gestation. This foetal isozyme has also been observed by Herzfeld and Greengard (1972) and Weinhouse *et al.* (1972a, 1972b).

In livers of human foetuses of 9 - 13 weeks gestational age, Benson and de Jong (1968) reported the presence of hexokinases I, IA, II and

III, as well as an additional band of intermediate mobility between HK II and HK III. This extra band was found to stain clearly in the presence of a high concentration of glucose but hardly at all at low glucose concentration. The foetal liver patterns reported by Rogers *et al.* (1975) differ somewhat from those reported by Benson and de Jong (1968). In the studies of Rogers *et al.* (1975), in which livers of foetuses of 17, 23 and 27 weeks gestation were examined, electrophoresis was performed in the absence of EDTA or mercaptoethanol and splitting of the bands was observed. In early foetal development single bands were found to be present in the regions corresponding to HK I, HK II and HK III. In the older foetuses extra more anodal bands were observed in these regions, the secondary band in each region becoming more pronounced with increasing age. Rogers *et al.* (1975) suggested that this sequential formation of secondary isozymes indicates evidence of *in vivo* modification of the primary enzyme. Human foetal muscle and heart have been found to contain only HK I (Benson and de Jong, 1968).

The HK isozyme patterns in experimental rat hepatomas differ from those of normal or host livers and in some cases resemble those of foetal liver. The regulatory isozyme HK IV is absent from fast-growing, poorly-differentiated hepatomas but is retained in the slow-growing, well-differentiated tumours (Sato *et al.*, 1969; Shatton *et al.*, 1969; Weinhouse *et al.*, 1972a; Sato and Sugimura, 1973). Sato and Sugimura (1973), who have observed splitting of HK IV in rat tissues, have shown that, whereas in normal liver HK IV_s is usually the predominant form, HK IV_f is present in some Morris hepatomas. Sato *et al.* (1969) observed decreases in the proportion of HK III and increases in the proportion of HK II in hepatomas of varying growth rates. Shatton *et al.* (1969), Weinhouse *et al.* (1972a) and Sato and Sugimura (1973) did not, however, report increases in the proportion of HK II in hepatomas.

In human hepatoma the HK isozyme pattern has been shown to

differ from that of normal liver, in that HK II is present and the proportion of HK III is decreased (Balinsky *et al.*, 1973a). Increases in the proportion of HK II have also been observed in human cervical and corpus carcinomas (Kikuchi *et al.*, 1972; Sato *et al.*, 1972c).

Rapidly-dividing rat liver cells, growing in culture, have been found to contain HK I and HK II, the liver-specific isozymes HK III and HK IV being absent (Walker *et al.*, 1972). Similarly, Yoshida ascites hepatoma cells and human liver cells were shown to have only HK I and HK II; in the human cells the proportion of HK II was found to increase in the presence of high concentrations of glucose (Katzen *et al.*, 1965; Sato *et al.*, 1972b). In hepatocytes derived from regenerated rat liver, however, the liver-specific isozyme HK IV was retained; this isozyme was not detected in the non-parenchymal cells (Bonney *et al.*, 1973).

Pyruvate kinase

The key glycolytic enzyme, pyruvate kinase (PK), catalyses the formation of pyruvate from phosphoenolpyruvate.

$$\text{phosphoenolpyruvate} + \text{ADP} \longrightarrow \text{pyruvate} + \text{ATP}$$

Pyruvate kinase multiplicity was first observed in rat and human tissues when two major isozymes were reported (Von Fellenberg *et al.*, 1963; Koler *et al.*, 1964; Tanaka *et al.*, 1965, 1967; Bigley *et al.*, 1968). It has since been established that three major forms of PK exist in mammalian tissues (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Walker and Potter, 1972; Whittell *et al.*, 1973; Osterman and Fritz, 1973; Osterman *et al.*, 1973; Farina *et al.*, 1974). These isozymes have been designated PK L (PK I, PK 1 or PK B), PK M_1 (PK M, PK II, PK 3 or PK A) and PK M_2 (PK III, PK 4 or PK C). (There is considerable confusion in the literature concerning the nomenclature of PK isozymes; the L, M_1

and M_2 nomenclature of Imamura and Tanaka (1972) will be used here). In all species so far examined, under the conditions used, PK L has a greater electrophoretic mobility than PK M_1 or PK M_2. The relative positions of PK M_1 and PK M_2 have, however, been shown to vary from one species to another and to depend on the pH at which electrophoresis is carried out, as well as on whether or not fructose-1,6-diphosphate is included in the buffers. A fourth type of PK of mobility slightly less than that of PK L is found in erythrocytes. It has been suggested by Imamura and Tanaka (1972) and Whittell *et al.* (1972) that this isozyme may be a hybrid of PK L and PK M_2, but the recent studies of Lincoln *et al.* (1975) have indicated that this is not the case. Additional minor bands between PK L and PK M_2 are found in mammalian kidney and occasionally in liver (Imamura and Tanaka, 1972; Whittell *et al.*, 1973). These intermediate bands in bovine kidney have been shown to be hybrid forms (Cardenas *et al.*, 1975). Similarly minor bands between PK M_1 and PK M_2 have occasionally been observed in some tissues (Whittell *et al.*, 1973). It seems likely on the basis of *in vitro* hybridisation studies and genetic investigations (Koler *et al.*, 1964; Susor and Rutter, 1971; Cardenas *et al.*, 1975) that the L, M_1 and M_2 isozymes are tetrameric structures containing genetically distinct subunits.

The PK isozyme patterns in normal adult rat tissues have been studied by means of cellulose acetate, polyacrylamide gel and starch gel or block electrophoresis and by means of ion-exchange chromatography (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Walker and Potter, 1972; Whittell *et al.*, 1973; Osterman and Fritz, 1973; Farina *et al.*, 1974). The only isozyme found in muscle is PK M_1. Liver contains PK L, the predominant form and PK M_2. PK M_2 is the predominant form in kidney which also has a small proportion of PK L and two or three minor bands between PK L and PK M_2. In both heart and brain, PK M_1 is the major isozyme and there are traces of

PK M_2. Intestine, thymus, spleen, stomach, lung, adipose tissue and testis all contain PK M_2. By means of isoelectric focusing techniques, the PK L and PK M_2 isozymes of rat tissues can be further separated into a number of different forms (Ibsen and Trippet, 1972; Ibsen *et al.*, 1975).

It is apparent that PK M_1 predominates in tissues such as muscle, heart and brain which are geared for rapid glycolysis whereas PK L, like HK IV, is restricted to the gluconeogenic tissues liver and kidney. Furthermore the activity of PK L is also influenced by diet and certain hormones whereas the activities of PK M_1 and PK M_2 are not (Krebs and Eggleston, 1965; Tanaka *et al.*, 1965, 1967). As mentioned previously, HK IV is restricted in its distribution to the parenchymal cells of rat liver and this is also the case for PK L as well as for fructose-1,6-diphosphatase and glucose-6-phosphatase, whereas non-parenchymal cells are characterised by the presence of PK M_2, HK I, HK II and HK III and small amounts of fructose-1,6-diphosphatase (Sapag-Hagar *et al.*, 1969; Crisp and Pogson, 1972; Van Berkel *et al.*, 1972). It is therefore apparent that two kinds of pathways for glycolysis, located in two different cell types, can be distinguished in rat liver. The hepatocytes respond to the metabolic changes of the liver and cater for gluconeogenesis as well as glycolysis whereas the non-parenchymal cells are almost entirely glycolytic.

Comparative studies of PK L, PK M_1 and PK M_2 from rat tissues have established that the three isozymes differ from each other kinetically (Imamura *et al.*, 1972). Both PK L and PK M_2 show sigmoidal kinetics with respect to phosphoenolpyruvate whereas PK M_1 shows Michaelis-Menten kinetics with respect to this substrate. The isozyme PK L is inhibited to a greater extent by ATP than PK M_1 or PK M_2; for PK L but not for PK M_1 or PK M_2 this inhibition is reversed by fructose-1,6-diphosphate. PK L and PK M_2 are both activated by fructose-1,6-diphosphate and glucose-6-phosphate whereas PK M_1 is not. Alanine is a strong inhibitor of both

PK L and PK M_2, although the mechanism appears to be different, since the inhibition of PK L is co-operative and completely reversed by addition of fructose-1,6-diphosphate whereas that of PK M_2 is not reversed by fructose-1,6-diphosphate. L-Phenylalanine inhibits PK L to a lesser extent than it inhibits PK M_2. Tryptophan also inhibits PK L and PK M_2. These amino-acids have little effect on PK M_1.

The studies of Imamura and Tanaka (1972) showed the distributions of the PK isozymes in human muscle, liver and spleen to be similar to those in the corresponding rat tissues. Muscle was found to contain PK M_1 and liver PK L and a trace of PK M_2. Osterman and Fritz (1973), however, did not distinguish clearly between PK M_1 and PK M_2 but showed that human muscle contained only PK M and that liver had almost equal proportions of PK L and PK M. Bigley *et al.* (1968) also found that human liver had a substantial proportion of PK M in addition to PK L, whereas Balinsky *et al.* (1973a) found that this tissue contained mainly PK L. Osterman and Fritz (1973) reported the presence of PK M in heart and of PK M together with two or three minor bands in kidney.

Comparative kinetic studies of human liver PK L and PK M (presumably PK M_2, although no distinction was made between the M_1 and M_2 isozymes) showed differences between the two forms (Balinsky *et al.*, 1973b). The Michaelis constants of PK L for phosphoenolpyruvate and ADP are higher than those of PK M. Moreover, the L isozyme shows allosteric kinetics with respect to phosphoenolpyruvate and substrate inhibition by ADP, whereas the M isozyme shows simple Michaelis-Menten kinetics with respect to phosphoenolpyruvate. The patterns obtained for steady-state kinetics and isotope exchange appear to indicate, however, that the catalytic mechanisms of human PK L and PK M are similar.

The PK isozyme patterns in foetal rat tissues have been found

to differ markedly from the adult patterns which are not acquired until after birth (Schapira and Gregori, 1971; Imamura and Tanaka, 1972; Walker and Potter, 1972; Osterman *et al.*, 1973; Schapira *et al.*, 1975). In foetal liver there are only traces of PK L, the major isozyme being PK M_2. In some cases an additional isozyme of mobility slightly slower than that of PK L has also been observed in liver. This isozyme has been shown to have a similar electrophoretic mobility to that of the erythrocyte isozyme and it probably represents the PK isozyme from foetal haemopoietic tissue. At birth the proportions of PK M_2 and the slower moving anodal band decrease and the proportion of PK L increases. Foetal muscle has mainly PK M_2 and the transition from PK M_2 to PK M_1 appears to commence just before birth. The major isozyme in foetal brain is PK M_2 and there are also traces of PK M_1. The only isozyme observed in heart and kidney before birth is PK M_2. Since the major isozyme in all foetal tissues examined is PK M_2, Imamura and Tanaka (1972) have suggested that PK M_2 should be regarded as the prototype of PK isozymes and both the M_1 and L forms as differentiated types since they increase during differentiation of tissues.

In experimental rat hepatomas the proportion of PK L decreases while that of PK M_2 increases, as compared with normal or host liver; this is suggestive of a trend towards the patterns in foetal or regenerating liver (Schapira and Gregori, 1971; Imamura and Tanaka, 1972; Walker and Potter, 1972; Weinhouse *et al.*, 1972a; Farina *et al.*, 1974; Yanagi *et al.*, 1974; Schapira *et al.*, 1975). The isozyme change in hepatomas appears to be related to growth rate. In slow-growing well-differentiated hepatomas a substantial proportion of PK L is retained whereas in fast-growing, poorly-differentiated tumours only the M_2 form is present. PK M_2 from Yoshida ascites hepatoma AH 130 and PK M_2 from liver have been shown to be similar with respect to Michaelis constants for phosphoenolpyruvate, activation by fructose-1,6-diphosphate and inhibition by ATP and alanine

(Imamura and Tanaka, 1972). By means of isoelectric focusing in a sucrose density gradient, the presence of a cancer-specific isozyme has been demonstrated in some hepatomas (Criss, 1969).

The replacement of PK L by PK M has been demonstrated in human hepatoma but no distinction was made between the M_1 and M_2 forms (Balinsky *et al.*, 1973a). PK M from human hepatoma and PK M from human foetal liver have been shown to differ in that hepatoma PK M displays complex kinetics with respect to phosphoenolpyruvate whereas the foetal liver isozyme displays simple Michaelis-Menten kinetics (Balinsky *et al.*, 1973b). The Michaelis constants for phosphoenolpyruvate were, however, found to be similar for both isozymes. Both forms were found to show Michaelis-Menten kinetics with respect to ADP and to have similar Michaelis constants for this substrate.

Spellman and Fottrell (1973) have reported the presence of an isozyme with slower electrophoretic mobility than PK M of liver or muscle in human lung, stomach and jejenum carcinomas. This isozyme was also observed in placenta.

In cultured cells derived from adult rat liver, PK M_2 was found to be present, the regulatory liver-specific isozyme PK L being absent (Walker *et al.*, 1972). Change in culture conditions was shown to affect the kinetic properties of the PK isozyme in these cells. The forms of the enzyme present in cells grown in the absence of glucose and in the presence of glucose appear to differ with respect to Michaelis constants for glucose, inhibition by ATP, alanine and phenylalanine and activation by fructose-1,6-diphosphate. Bonney *et al.* (1973) have, however, shown that in parenchymal cells derived from regenerated rat liver PK L is the major isozyme; in non-parenchymal cells PK M_2 is the major form. These workers also found that in hepatocytes derived from regenerating liver, the proportion of PK M_2 increases while that of PK L decreases; as

regeneration proceeds the adult pattern is regained.

Lactate dehydrogenase

The bifunctional enzyme, lactate dehydrogenase (LDH), catalyses the reversible conversion of pyruvate to lactate.

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

Wieland and Pfleiderer (1957) and Markert and Møller (1959) first demonstrated the presence of five electrophoretically distinct forms of LDH in animal tissues. These forms have been designated LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5 in order of decreasing electrophoretic mobility towards the anode. The studies of Wieland, Markert and Kaplan and co-workers have shown that the different molecular forms of LDH arise from the combination of two distinct types of protein subunit to form active tetrameric structures (Wieland and Pfleiderer, 1957; Markert and Møller, 1959; Appella and Markert, 1961; Cahn *et al.*, 1962; Fine *et al.*, 1963; Markert, 1963; Kaplan, (1964). The two types of subunit have been designated M or A (predominant in muscle) and H or B (predominant in heart). LDH_1 consists of four H subunits and LDH_5 consists of four M subunits; the intermediate isozymes LDH_2, LDH_3 and LDH_4 comprise subunits of both types (LDH_2 corresponds to H_3M, LDH_3 to H_2M_2 and LDH_4 to HM_3). The distribution of the LDH isozymes in a particular tissue depends on the proportions of H and M subunits present. A binomial distribution is found when the two types of subunit are present in equal amounts.

Hybridisation of LDH isozymes *in vitro* was first demonstrated by Markert (1963). It was found that freezing a mixture of LDH_1 and LDH_5 in concentrated salt solution gave rise to all five isozymes, presumably as a result either of dissociation and random recombination of the subunits or of subunit exchange between the isczymes. Subsequently, Chilson *et al.*

(1965) carried out detailed studies of the effects of freezing on hybridisation of LDH isozymes. The process was found to be complex and to be affected by factors such as rapidity of freezing and thawing, salt and protein concentration during freezing, decreased pH near the eutectic point and specific ion effects. From the relative proportions of the different isozymes formed during hybridisation procedures and the physical and chemical treatment required to effect hybridisation, considerable information has been obtained regarding the strength and type of interaction and the degree of fit between different subunits (Vesell, 1965; Chilson *et al.*, 1965). Hybridisation studies of isozymes from two different species have been extensively carried out (Chilson *et al.*, 1965; Fondy and Kaplan, 1965; Vesell, 1965; Salthe *et al.*, 1965). Even though species differences in the nature of the subunits are apparent (as indicated by the formation of hybrid isozymes with mobilities different from those present in tissues of the species examined), hybridisation takes place between isozymes from a wide variety of species, indicating that during evolution the features required for association of the subunits into active tetramers are retained.

In addition to having different electrophoretic mobilities, the LDH_1 and LDH_5 isozymes from a number of species have been shown to differ with respect to amino acid composition, immunological characteristics and catalytic properties (Cahn *et al.*, 1962; Markert and Ursprung, 1962; Fine *et al.*, 1963; Kaplan and White, 1963; Nisselbaum and Bodansky, 1963; Pesce *et al.*, 1964; Kaplan, 1964; Vesell, 1965). The properties of LDH_2, LDH_3 and LDH_4 are as might be predicted from their relative proportions of H and M subunits.

Differences in LDH isozyme patterns in various normal adult rat tissues have been demonstrated by means of starch gel electrophoresis (Fine *et al.*, 1963; Kline and Clayton, 1964; Johnson and Kampschmidt,

1965). In liver and muscle, LDH_5 is the major isozyme. Occasionally a trace of LDH_4 is also detected in liver and small amounts of LDH_4, LDH_3 and LDH_2 may be present in muscle. In heart and kidney, LDH_1 is the predominant form and varying amounts of the other four isozymes are also present. By means of isoelectric focusing in a sucrose density gradient, a pI value of 8.6 has been obtained for rat muscle LDH_5 (Weller *et al.*, 1973).

In human tissues all five LDH isozymes are usually observed on electrophoresis (Vesell and Bearn, 1961; Vesell, 1965). In liver, muscle, skin and lung, LDH_5 is the major isozyme and the proportions of LDH_4, LDH_3, LDH_2 and LDH_1 decrease with increasing mobility towards the anode. LDH_1 is the predominant form in heart and kidney with the proportions of LDH_2, LDH_3, LDH_4 and LDH_5 decreasing in order of decreasing mobility. On isoelectric focusing in a sucrose density gradient, a single major LDH_5 component has been observed in human muscle and found to have a pI of 9.4 (Weller *et al.*, 1973). Latner (1973) and Fukuda *et al.* (1974) found the LDH isozyme patterns obtained on disc gel isoelectric focusing to be different from the electrophoretic patterns and observed more than five bands of activity in human tissue extracts and serum. It has, however, been suggested that the additional bands may result from the formation of enzyme complexes with Ampholines or with tissue proteins or be due to the presence of varying amounts of coenzyme (Latner, 1973).

In general the LDH isozyme pattern is very constant for a particular tissue of a given species and is not affected by dietary or hormonal factors (Markert and Sladen, 1966).

As in other species, the human LDH_1 and LDH_5 isozymes differ with regard to affinity for pyruvate and lactate and inhibition by these substrates (Pfleiderer and Wachsmuth, 1961; Vesell, 1965). The Michaelis

constants of LDH_5 for pyruvate and lactate are somewhat higher than those of LDH_1. For both isozymes, however, the Michaelis constants for lactate are higher than for pyruvate. One of the main functions of LDH is to regulate the intracellular ratio of NAD^+ to NADH. This ratio is maintained above 1, both by the establishment of an enzyme-independent equilibrium constant for the reaction which is ten times more favourable for NAD^+ than for NADH formation, as well as by the greater affinity of the LDH isozymes for pyruvate than for lactate. Since the LDH isozymes vary in affinity for these two substrates, each isozyme may serve to develop slightly different ratios of NAD^+ to NADH. LDH_1 is strongly inhibited by relatively low levels of lactate whereas LDH_5 is unaffected even at high concentrations of this substrate. Pyruvate inhibits both LDH_1 and LDH_5 but LDH_1 is affected to a greater extent. This may explain the predominance of LDH_5 in anaerobic tissues such as liver and muscle which are orientated towards lactate formation during glycolysis. LDH_1 predominates in aerobic tissues such as brain and heart where lactate accumulation is minimal.

In foetal rat tissues, as in foetal mouse, rabbit and bovine tissues, there is a predominance of M subunit-containing isozymes (Markert and Ursprung, 1962; Fine *et al.*, 1963). The pattern in foetal rat muscle is similar to that in adult muscle and there is little change during development. There is a slightly greater proportion of LDH_4 in foetal liver than in adult liver, the amount decreasing just after birth. In foetal heart and kidney, LDH_4 and LDH_5 are present initially and the proportions of LDH_1, LDH_2 and LDH_3 increase gradually during development.

In contrast, there is a high proportion of H subunit-containing isozymes in human foetal tissues as well as in tissues of the foetal chicken (Pfleiderer and Wachsmuth, 1961; Fine *et al.*, 1963; Dawson *et al.*, 1964; Dawson and Kaplan, 1965). The patterns in a wide variety of human foetal tissues including muscle, liver, heart, brain, lung and

kidney are similar, with LDH_3 being the predominant isozyme from an early stage. Slight increases in the proportion of H subunits are observed in foetal muscle and liver just before birth.

Increases in the proportion of H subunit-containing isozymes, suggestive of a trend towards the foetal liver pattern, have been observed in some rat hepatomas (Kline and Clayton, 1964; Johnson and Kampschmidt, 1965; Schapira and de Néchaud, 1968; Rosado *et al.*, 1969). In five out of ten hepatomas studied, Schapira and de Néchaud (1968) reported the presence of LDH_2 and LDH_3 in addition to LDH_4 and LDH_5, the forms present in normal or host liver. These workers also detected an abnormal band intermediate between LDH_3 and LDH_4 in one hepatoma; this isozyme was also found to occur in foetal liver (Schapira and de Néchaud, 1968). Rosado *et al.* (1969) did not find any correlation between LDH isozyme patterns and growth rate or degree of differentiation in a series of rat hepatomas. In all cases there was found to be a predominance of M subunits, occasionally no H subunits were detected and in a few cases, the H subunit proportion was greater than in normal liver. In other rat tumours (kidney and mammary) increases in the proportion of M subunits, which may be related to the anaerobic conditions prevailing in these tissues, have been reported (Rosado *et al.*, 1969; Schweitzer *et al.*, 1972).

The LDH isozyme patterns in human hepatomas frequently resemble those of normal liver, although in some cases slight increases in the proportion of H subunits have been observed (Balinsky *et al.*, 1973a). During studies of a wide variety of non-hepatic human tumours, some workers have occasionally observed increases in the proportion of H subunits, but in the majority of cases increases in the proportion of M subunits have been reported (Starkweather and Schoch, 1962; Goldman and Kaplan, 1963; Goldman *et al.*, 1964; Langvad, 1972).

Tissue-specific LDH isozyme patterns are frequently lost in culture. Cell cultures derived from embryonic chicken tissues, rabbit tissues, human embryo liver and kidney, adult liver, heart, intestine and skin show a predominance of M subunits which is possibly related to the anaerobic environment prevailing in culture (Nitowsky and Soderman, 1964; Vesell, 1965). By decreasing the oxygen tension during growth, Fondy and Kaplan (1965) found that an increase in the proportion of M subunits occurred in chicken embryo heart and muscle, newborn mouse kidney and monkey heart cells. Nitowsky and Soderman (1964) did not observe any correlation between isozyme patterns and changes in nutrient medium in human cells. German *et al.* (1964), however, reported an increase in the proportion of H subunits in HeLa cells grown in the absence of serum.

PHOSPHOENOLPYRUVATE CARBOXYKINASE

The occurrence, subcellular distribution and regulatory features of phosphoenolpyruvate carboxykinase (PEPCK) have been discussed in the previous section (see p. 8). In this section, the physical and kinetic properties of the enzyme obtained from various sources will be considered.

Reactions catalysed

The enzyme PEPCK found in plant and animal species catalyses reaction (1).

$$\text{phosphoenolpyruvate} + CO_2 + \text{NDP} \underset{\longleftarrow}{\overset{\text{Metal}^{2+}}{\longrightarrow}} \text{oxaloacetate} + \text{NTP} \quad (1)$$

NDP and NTP represent nucleoside di- and triphosphates, respectively. In vertebrate tissues the reaction involves inosine or guanine nucleotides, whereas in yeast and some species of bacteria it involves adenine nucleotides (Utter and Kolenbrander, 1972). As indicated, the reaction is reversible; the equilibrium constant is 2.69 at 30° (Utter and Kurahashi, 1954b). The

formation of phosphoenolpyruvate is an essential step in the synthesis of glucose and glycogen from pyruvate, amino acids or citric acid cycle intermediates.

In addition to reaction (1), PEPCK also catalyses the irreversible decarboxylation of oxaloacetate to pyruvate as shown in reaction (2).

$$\text{oxaloacetate} \xrightarrow{\text{NDP, NTP}} \text{pyruvate} + CO_2 \quad (2)$$

In vertebrate tissues the reaction requires IDP or GDP (Utter and Kurahashi, 1954b; Chang and Lane, 1966; Noce and Utter, 1975) whereas in yeast it requires ADP or ATP (Cannata and Stoppani, 1963). The physiological significance of reaction (2) is not clear at present.

The highly purified enzyme from yeast also possesses weak pyruvate kinase activity in that it catalyses reaction (3) (Cannata and Stoppani, 1963).

$$\text{phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Metal}^{2+}} \text{pyruvate} + \text{ATP} \quad (3)$$

In this work reaction (1) was of interest and therefore reactions (2) and (3) will not be considered further.

Enzyme assays

The activity of PEPCK in reaction (1) can be determined either in the direction of oxaloacetate formation (carboxylation) or in the physiological direction of phosphoenolpyruvate synthesis (decarboxylation). As a result of the absence of any simple, generally applicable, continuous assay system, a number of widely differing 'stopped' procedures have been developed. In the carboxylation direction, the most widely used method is that developed by Chang and Lane (1966) and modified by Ballard and Hanson (1967), which involves measurement of ^{14}C-incorporation into oxaloacetate. A method used frequently for determination of activity in the decarboxylation

direction is that of Seubert and Huth (1965), in which phosphoenolpyruvate formed during the reaction is determined spectrophotometrically. A more sensitive decarboxylation assay, involving transfer of ^{32}P from GTP to phosphoenolpyruvate, has been developed by Walsh and Chen (1971). In the decarboxylation direction, problems are encountered as a result of non-enzymic breakdown of oxaloacetate to pyruvate making it difficult to determine true substrate concentrations. Consequently, Meyuhas *et al.* (1971) have developed methods in which oxaloacetate is generated *in situ* in the reaction mixture from malate or aspartate. Continuous spectrophotometric methods, only suitable when using purified preparations, have been developed by Chang and Lane (1966). For the continuous carboxylation and decarboxylation assays the enzyme must be free from pyruvate kinase and malate dehydrogenase activities, respectively. The decarboxylation assay also has the disadvantage that there is a blank reaction due to breakdown of oxaloacetate to pyruvate. A continuous decarboxylation assay based on the method of Meyuhas *et al.* (1971) has been used by Jomain-Baum *et al.* (1976). The formation of phosphoenolpyruvate is measured by equilibrium displacement in a system in which oxaloacetate is generated from malate (at non-inhibitory concentrations) by malate dehydrogenase.

Enzyme activities and therefore the relative rates of the oxaloacetate- and phosphoenolpyruvate-forming reactions vary depending on the assay used. Pogson and Smith (1975) showed that activities determined using the method of Seubert and Huth (1965) are approximately 4-fold higher than those determined using the method of Walsh and Chen (1971). For rat liver PEPCK, they found the ratios of activity in the carboxylation direction to activity in the decarboxylation direction to be 0.6 and 3.7 when using the methods of Seubert and Huth (1965) and Walsh and Chen (1971), respectively (the radioactive carboxylation assay (Ballard and Hanson, 1967) was used in each case). A value for this ratio of approximately 0.09, obtained using

continuous spectrophotometric assays, has been reported for the enzyme from pig liver (Chang *et al.*, 1966).

Enzyme purification

PEPCK has been purified from yeast (Cannata, 1970), chicken liver mitochondria (Utter and Kurahashi, 1954a; Kurahashi *et al.*, 1957; Felicioli *et al.*, 1970; Noce and Utter, 1975), pig liver mitochondria (Chang and Lane, 1966), sheep kidney mitochondria (Barns and Keech, 1972), sheep liver cytosol and mitochondria (Bandurski and Lipmann, 1956; Ballard, 1970), guinea pig liver cytosol and mitochondria (Holten and Nordlie, 1965) and rat liver cytosol (Ballard and Hanson, 1969; Ballard, 1970; Philippidis *et al.*, 1972). The purification procedures used have included ammonium sulphate fractionation, treatment with calcium phosphate gel, Sephadex, DEAE-Sephadex, DEAE-cellulose, cellulose phosphate and hydroxylapatite chromatography and isoelectric focusing. The most highly purified enzyme preparations have been obtained from yeast (in crystalline form) (Cannata, 1970), rat liver cytosol (380 fold, 15% yield) (Ballard and Hanson, 1969), pig liver mitochondria (208 fold, 48% yield) (Chang and Lane, 1966) and chicken liver mitochondria (140 fold, 3.2% yield) (Felicioli *et al.*, 1970). For other preparations purification factors range from 7 - 100 fold.

During the purification of the enzyme from rat, guinea pig, sheep, pig and chicken livers by the workers mentioned above, only a single form of the enzyme was found to be present in either the cytosol or mitochondrial fractions. Additional forms (one in the cytosol and one in the mitochondria), separable on DEAE-Sephadex have, however, been observed in chicken embryo and young chicken livers (Jo *et al.*, 1974). These additional forms were also found to be present in adult livers after starvation and following administration of hormones.

Physical properties

The molecular weights of PEPCK isolated from vertebrate tissues are of the order 70,000. The value for the pig liver enzyme, determined by sedimentation equilibrium, is 73,300 (Chang and Lane, 1966). The values for the enzymes from rat and chicken liver, determined by gel filtration and electrophoretic techniques, are 74,500 (Ballard and Hanson, 1969) and 68,000 (Utter and Kolenbrander, 1972), respectively. Using sedimentation-diffusion analysis, Barns and Keech (1972) estimated the molecular weight of sheep kidney mitochondrial PEPCK to be 71,100. In contrast, values of 130,000 and 252,000 have been obtained for the enzymes from *Bacillus stearothermophilus* and yeast, respectively (Cannata, 1970). It is possible that the vertebrate enzyme is a monomer, the *Bacillus stearothermophilus* enzyme a dimer and the yeast enzyme a tetramer, although this had not yet been substantiated.

By means of isoelectric focusing in a sucrose density gradient, a value 5.04, indicative of an excess of acidic amino acid residues, has been obtained for the isoelectric point of rat liver PEPCK (Ballard and Hanson, 1969).

Kinetic properties

Much of the information concerning the kinetic properties of PEPCK has been obtained from studies of the enzymes from chicken, pig, guinea pig and rat liver. Table II summarises some of the properties of PEPCK from these sources.

Differences between the cytosol and mitochondrial enzymes

The differences in subcellular distribution patterns of PEPCK in different species and the variations in response of the cytosol and mitochondrial forms to hormonal and metabolic changes (see p. 10) suggested

TABLE IIA

PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM LIVERS OF DIFFERENT SPECIES

CARBOXYLATION (OXALOACETATE FORMATION) REACTION

PROPERTY	CHICKEN LIVER MITOCHONDRIA	PIG LIVER MITOCHONDRIA	GUINEA PIG LIVER MITOCHONDRIA	GUINEA PIG LIVER CYTOSOL	RAT LIVER CYTOSOL
Relative activities with nucleoside diphosphates (%)		IDP 100 GDP 109 ADP 0 (Chang and Lane, 1966)			IDP 100 GDP 104 UDP 1.2 ADP 0.9 CDP 0.3 (Ballard and Hanson, 1967)
Optimal pH	6.2 (Utter and Kolenbrander, 1972)	6.6 (Chang *et al.*, 1966)	7.4 (Holten and Nordlie, 1965)	7.4 (Holten and Nordlie, 1965)	7.0 (Ballard and Hanson, 1967)
Metal ion specificity		Active with Mn^{2+} Only slight activity with Mg^{2+} (Chang and Lane, 1966)	Active with Mn^{2+} Negligible activity with Mg^{2+}, Ca^{2+} or Zn^{2+} (Holten and Nordlie, 1965)	Active with Mn^{2+} Negligible activity with Mg^{2+}, Ca^{2+} or Zn^{2+} (Holten and Nordlie, 1965)	
Apparent Michaelis constants (mM)					
Phosphoenolpyruvate		0.12, 0.29 (Chang *et al.*, 1966) 0.6 (Swiatek *et al.*, 1970)	0.13 (Holten and Nordlie, 1965)	0.14 (Holten and Nordlie, 1965)	0.4 (Ballard and Hanson, 1967)
Bicarbonate		16, 25 (Chang *et al.*, 1966) 15 (Swiatek *et al.*, 1970)			
IDP		0.033, 0.098 (Chang *et al.*, 1966) 0.054 (Swiatek *et al.*, 1970)	0.07, 0.19 (Holten and Nordlie, 1965)	0.16, 0.62 (Holten and Nordlie, 1965)	
GDP		0.063 (Chang *et al.*, 1966)			

TABLE IIB

PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM LIVERS OF DIFFERENT SPECIES

DECARBOXYLATION (PHOSPHOENOLPYRUVATE FORMATION) REACTION

PROPERTY	CHICKEN LIVER MITOCHONDRIA	PIG LIVER MITOCHONDRIA	GUINEA PIG LIVER MITOCHONDRIA	GUINEA PIG LIVER CYTOSOL	RAT LIVER CYTOSOL
Relative activities with nucleoside triphosphates (%)		GTP 100 ITP 79 UTP 23 CTP 0 ATP 0 (Chang and Lane, 1966)	ITP 100 GTP 52 (Nordlie and Lardy, 1963)		
Optimal pH	7.0 - 8.0 (Utter and Kolenbrander, 1972)	6.6 - 8.0 (Chang *et al.*, 1966)	8.0 - 8.4 (Holten and Nordlie, 1965)	8.2 (Holten and Nordlie, 1965)	7.3 - 8.1 (Foster *et al.*, 1967)
Metal ion specificity		Active with Mn^{2+} Slight activity with Mg^{2+} (Chang and Lane, 1966)	Activated by Mn^{2+} and Mg^{2+} and to a lesser extent by Fe^{2+} and Co^{2+} (Holten and Nordlie, 1965)	Activated by Mn^{2+} and Mg^{2+} and to a lesser extent by Fe^{2+} and Co^{2+} (Holten and Nordlie, 1965)	Active with Mn^{2+} and Mg^{2+} (Foster *et al.*, 1967)
Apparent Michaelis constants (mM)					
Oxaloacetate	0.009, 0.022 (Ballard, 1970) 4.0 (Felicioli *et al.*, 1970)	0.14 (Chang *et al.*, 1966)	2.5 (Holten and Nordlie, 1965)	0.9 (Holten and Nordlie, 1965)	0.0015 - 0.005 (Walsh and Chen, 1971) 0.009, 0.019 (Jomain-Baum *et al.*, 1976) 0.022, 0.3 (Meyuhas *et al.*, 1971) 0.026, 0.029 (Ballard, 1970) 0.14 (Foster *et al.*, 1967) 0.15 (Marco and Sols, 1970)
ITP	2.9 (Felicioli *et al.*, 1970)	0.58 (Chang *et al.*, 1966)	0.0015 (Walsh and Chen, 1971) 0.9 - 1.8 (Holten and Nordlie, 1965)	0.004 (Walsh and Chen, 1971) 0.6 - 1.5 (Holten and Nordlie 1965)	0.005 (Walsh and Chen, 1971)
GTP		0.16 (Chang *et al.*, 1966)	0.0015 (Walsh and Chen, 1971) 1.4 (Holten and Nordlie, 1965)	0.005 (Walsh and Chen, 1971) 1.4 (Holten and Nordlie 1965)	0.005 (Walsh and Chen, 1971) 0.016, 0.022 (Jomain-Baum *et al.*, 1976) 0.06 (Foster *et al.*, 1967)
Inhibitors	p-Hydroxymercuribenzoate N-Ethylmaleimide Sodium tetrathionate (Utter and Kolenbrander, 1972) IDP (Felicioli *et al.*, 1970) Malate (Ballard, 1970)	p-Chloromercuribenzoate N-Ethylmaleimide Potassium fluoride (Chang and Lane, 1966) GDP GMP Bicarbonate (Chang *et al.*, 1966)	Quinolinate (Robinson and Oei, 1975) 3-Mercaptopicolinate (Robinson and Oei, 1975) AMP (Holten and Nordlie, 1965)	Quinolinate (Robinson and Oei, 1975) 3-Mercaptopicolinate (Robinson and Oei, 1975)	Quinolinate (Snoke *et al.*, 1971) 3-Mercaptopicolinate (Jomain-Baum *et al.*, 1976) Hydrazine (Ray *et al.*, 1970) α-Glycerophosphate (Foster *et al.*, 1967) Acetaldehyde (Baxter, 1976) Malate (Ballard, 1970)

the possibility of differences between the two forms.

In an attempt to resolve different metabolic functions of cytosol and mitochondrial PEPCK, Holten and Nordlie (1965) undertook a comparative study of the two enzymes from guinea pig liver. This work, however, showed that there are only slight differences between the two forms. In the direction of phosphoenolpyruvate synthesis at pH 8.0, with metal-ion concentrations exceeding 4 mM, the mitochondrial enzyme is more active with magnesium than with manganese, whereas the cytosol form is more active with manganese than with magnesium. The mitochondrial enzyme is inhibited by 1.33 mM AMP, whereas the cytosol form is unaffected. The two enzymes are similar with respect to pH optima in both directions, synergistic effects of manganese plus magnesium in the direction of phosphoenolpyruvate synthesis, absolute dependence on manganese in the direction of oxaloacetate synthesis and apparent Michaelis constants for phosphoenolpyruvate, IDP, oxaloacetate, ITP and GTP, assayed under a variety of conditions. Holten and Nordlie (1965) suggested that the cytosol and mitochondrial carboxykinases could be variants of the same enzyme protein and that the observed differences in metal-ion activation and AMP inhibition could be reflections of subtle differences in the secondary and tertiary structure of the enzyme, which in turn could be influenced by variations in composition of the original subcellular environment of the enzyme. They concluded that the marked similarities between the two forms preclude the assignment of different metabolic functions to the cytosol and mitochondrial carboxykinases solely on the basis of catalytic properties.

The studies of Ballard and Hanson (1967) showed that cytosol PEPCK from adult rat liver and mitochondrial PEPCK from foetal rat liver are similar with respect to relative velocities with IDP, GDP, UDP, ADP and CDP, pH optima in the carboxylation direction and apparent Michaelis constants for phosphoenolpyruvate. These two enzymes have, however, been

shown to differ immunologically (Ballard and Hanson, 1969).

Differences between cytosol and mitochondrial PEPCK with respect to apparent Michaelis constants for oxaloacetate and inhibition by malate have been demonstrated by Ballard (1970). In the presence of manganese, under the conditions used, the cytosol enzymes from rat and sheep liver both have Michaelis constants for oxaloacetate of the order 0.025 mM, whereas the mitochondrial enzymes from sheep and chicken liver both have Michaelis constants of the order 0.009 mM for this substrate. In the presence of magnesium instead of manganese, however, the Michaelis constants of all four enzymes for oxaloacetate are similar (0.02 - 0.03 mM). Ballard (1970) suggested that, although manganese is known to be present in mitochondria at low concentrations, the higher stability constants for Mn^{2+} - ITP and Mn^{2+} - GTP, as compared with the respective Mg^{2+} complexes, could indicate that a significant proportion of these nucleotides are present as Mn^{2+} complexes, in which case the lower Michaelis constants of mitochondrial PEPCK in the presence of manganese may be of physiological significance. The lower Michaelis constants of mitochondrial PEPCK are consistent with the lower oxaloacetate concentrations found in the mitochondria as compared with the cytosol (Williamson *et al.*, 1968, 1969). Malate inhibits the rat liver cytosol, sheep liver cytosol and mitochondrial and chicken liver mitochondrial enzymes to about the same extent at low oxaloacetate concentrations, in the presence of either manganese or magnesium. At high oxaloacetate concentrations, the inhibition is not evident for the mitochondrial enzymes in the presence of either metal, whereas the cytosol enzymes are inhibited in the presence of magnesium but not in the presence of manganese. The differences in malate inhibition, although interesting from a kinetic standpoint, are unlikely to be of physiological significance since the inhibitor constant is well in excess of malate concentrations *in vivo*.

Differences between the cytosol and mitochondrial enzymes of guinea pig liver with respect to inhibition by 3-mercaptopicolinate have recently been noted by Robinson and Oei (1975).

Nucleoside phosphate specificity

In general, adenine nucleotides are required for the enzyme from bacterial species and plants and inosine or guanine nucleotides for the enzyme from animal tissues (Utter and Kolenbrander, 1972). The enzyme from the protozoan *Tetahymena pyriformis* may be an intermediate; adenosine derivatives are more effective than those of inosine or guanine, although the latter are also able to function. A similar case may also exist for the enzyme from *Bacillus stearothermophilus*, which responds to adenine, guanine, thymidine and uridine phosphates in that order.

In the carboxylation direction, the enzymes from pig liver mitochondria and rat liver cytosol have similar activities with GDP or IDP (Chang and Lane, 1966; Ballard and Hanson, 1967). For sheep kidney mitochondrial PEPCK, the activity with GDP is slightly lower than with IDP (Barns and Keech, 1972). In the decarboxylation direction, the pig liver mitochondrial enzyme is more active with GTP than with ITP, whereas the enzymes from guinea pig liver and sheep kidney mitochondria show maximal activity with ITP (Nordlie and Lardy, 1963; Chang and Lane, 1966; Barns and Keech, 1972).

pH Optima

It is apparent from the values given in Table II, that the pH optima for the carboxylation reaction are in general lower than for the decarboxylation reaction. In all cases, the alkaline slopes for the carboxylation reaction are steeper than for the decarboxylation reaction (Utter *et al.*, 1954; Holten and Nordlie, 1965; Chang *et al.*, 1966; Utter

and Kolenbrander, 1972). It has been suggested (Utter and Kolenbrander, 1972) that the differences in pH optima for the two reactions may reflect the availability of the substrate, carbon dioxide; as the pH increases the actual concentration of carbon dioxide at any fixed concentration of bicarbonate will decrease.

The pH optima for the guinea pig liver enzymes in both directions are somewhat higher than those for chicken or pig liver mitochondrial PEPCK (Utter *et al.*, 1954; Holten and Nordlie, 1965; Chang *et al.*, 1966). Barns and Keech (1972) have demonstrated differences in optimum pH depending on the metal ion used; in the carboxylation direction the sheep kidney mitochondrial enzyme has a higher pH optimum in the presence of magnesium (7.5) than in the presence of manganese (6.5). However, it is not known whether similar effects occur in other species.

Metal-ion activation

In all species examined PEPCK has an absolute requirement for a divalent metal ion. The most effective cation is usually manganese, although other divalent ions may often substitute for it with varying degrees of efficiency.

In the direction of oxaloacetate synthesis, the pig liver and sheep kidney mitochondrial enzymes are activated by manganese and to a much lesser extent by magnesium (Chang and Lane, 1966; Barns and Keech, 1972). At pH 7.3, the activity of pig liver PEPCK with 2 mM magnesium chloride is negligible whereas at pH 7.5 it is 16% of the activity with 1.0 mM manganese chloride. In this direction the cytosol and mitochondrial enzymes from guinea pig liver both show an absolute requirement for manganese which can not be replaced by magnesium, calcium or zinc (Holten and Nordlie, 1965).

In the direction of phosphoenolpyruvate formation, the pig liver and sheep kidney mitochondrial enzymes are again considerably more active with manganese than with magnesium; for pig liver PEPCK the activity with 1.0 mM magnesium chloride is 6% of that with 1.25 mM manganese chloride (Chang and Lane, 1966; Barns and Keech, 1972). The cytosol and mitochondrial enzymes of guinea pig liver are activated by manganese and magnesium and to a lesser extent by iron and cobalt (Holten and Nordlie, 1965). At pH 7.3 both forms are more active with manganese than with magnesium, whereas at pH 8.0 and metal-ion concentrations exceeding 4 mM, the mitochondrial enzyme is more active with magnesium than with manganese. The guinea pig liver enzymes both show maximal activity when the manganese chloride:ITP ratios are 1:2 and are inhibited at manganese concentrations exceeding the optimal values. The mitochondrial enzyme shows maximal activity with magnesium chloride:ITP ratios of between 2:1 and 3:1 and there is no inhibition by excess metal. The cytosol enzyme reaches maximal activity when the magnesium chloride:ITP ratio is 1:1 and at higher magnesium concentrations is inhibited. Rat liver cytosol PEPCK is activated by manganese and magnesium, the activity with magnesium chloride being 88% of that with manganese chloride at optimal concentrations of both metals (Foster *et al.*, 1967). The activity is maximal with manganese chloride, acetate or sulphate:ITP ratios of 1:2 and the enzyme is inhibited by excess metal, the extent of inhibition being greater in the presence of manganese chloride or acetate than in the presence of manganese sulphate. Magnesium chloride, acetate or sulphate:ITP ratios of approximately 1:1 are required for maximal activity and there is slight inhibition in the presence of high concentrations of magnesium chloride but not in the presence of magnesium acetate or sulphate.

Synergistic effects of manganese and magnesium have been observed for the decarboxylation reaction. With both the cytosol and mitochondrial

enzymes from guinea pig liver the presence of magnesium and manganese together results in enhanced activity, the effects of the two ions being almost additive (Holten and Nordlie, 1965). Increased activity in the presence of both metal ions has also been reported for the chicken, pig and rat liver enzymes (Chang and Lane, 1966; Foster *et al.*, 1967; Utter and Kolenbrander, 1972). For rat liver cytosol PEPCK, maximal activity is obtained using a high ratio of magnesium:manganese (Foster *et al.*, 1967). It therefore seems likely that the function of magnesium is to form a $MgITP^{2-}$ complex which acts as a substrate in the reaction; the low concentration of manganese required for maximal activity, when substrate concentrations of $MgITP^{2-}$ are present, suggests that manganese combines with the enzyme rather than with one of the substrates or products of the reaction.

Studies of the activity of rat liver cytosol PEPCK in the decarboxylation direction, in the presence of a fixed concentration of magnesium and varying concentrations of other metal ions, have been carried out by Snoke *et al.* (1971). Divalent manganese, cadmium, cobalt and iron salts increase the activity, whereas silver, aluminium, barium, caesium, calcium, lanthanum, lithium, mercury, nickel, strontium and zinc salts are without effect. Iron (III) is also without effect.

Apparent Michaelis constants

The Michaelis constants of pig, guinea pig and rat liver PEPCK for phosphoenolpyruvate are of a similar order of magnitude and fall within the range 0.1 to 0.6 mM (Holten and Nordlie, 1965; Chang *et al.*, 1966; Ballard and Hanson, 1967; Swiatek *et al.*, 1970). Physiological concentrations of phosphoenolpyruvate in rat liver have been reported to be 0.04 - 0.12 mM (Hornbrook *et al.*, 1965).

High values (15 - 25 mM) have been reported for the Michaelis constants of pig liver mitochondrial PEPCK for bicarbonate (Chang *et al.*, 1966; Swiatek *et al.*, 1970), indicating that at physiological concentrations of bicarbonate (Long (1961) has reported a concentration of 20 mM for mammalian plasma), the enzyme would be only half-saturated. Although the bicarbonate ion was originally considered to be the active species involved in the reaction (Chang *et al.*, 1966; Maruyama *et al.*, 1966), it now appears that carbon dioxide is the actual substrate (Cooper *et al.*, 1968) and it would therefore be preferable to express the Michaelis constants in terms of carbon dioxide concentration. The equilibrium proportions of carbon dioxide and bicarbonate ion in bicarbonate solutions at a particular pH can be determined from the Henderson-Hasselbalch equation (see Jencks and Regenstein, 1973).

The Michaelis constants of pig liver mitochondrial PEPCK for IDP, determined in the presence of different concentrations of phosphoenolpyruvate and manganese, range from 0.033 to 0.098 mM; the value for GDP is also within this range (Chang *et al.*, 1966; Swiatek *et al.*, 1970). For both the cytosol and mitochondrial enzymes of guinea pig liver, the Michaelis constants for IDP are higher (particularly so for the cytosol enzyme) when the $MnCl_2$:IDP ratio is maintained at 2:1 (0.62 and 0.19 mM, respectively) than when the manganese chloride concentration is maintained at a constant value of 1.2 mM (0.16 and 0.07 mM, respectively) (Holten and Nordlie, 1965). The intracellular concentration of GDP in rat liver is of the order 0.1 - 0.2 mM (Clifford *et al.*, 1972).

It can be seen from Table II that the values reported for the Michaelis constants of PEPCK for oxaloacetate vary over a wide range. For the chicken liver enzyme, the value reported by Felicioli *et al.* (1970) is almost 200 times greater than that reported by Ballard (1970). The values reported for rat liver cytosol PEPCK vary from 0.0015 to 0.15 mM,

a difference of 100-fold. The physiological concentration of oxaloacetate in rat liver is of the order 0.1 to 10 μM (Williamson *et al.*, 1968, 1969) and many of the values reported for the Michaelis constants for this substrate are not consistent with this or with the observed rates of glucose synthesis (Scrutton and Utter, 1968). The lowest values have been obtained by Walsh and Chen (1971). These workers, however, used crude enzyme preparations and suggested that some of the higher values obtained by others may have resulted from denaturation or removal of a cofactor during purification procedures. Meyuhas *et al.* (1971) also used crude preparations but did not obtain values as low as those of Walsh and Chen (1971).

It is unlikely that such wide variations in Michaelis constants for oxaloacetate can be accounted for by the differences in pH, metal ion and ITP concentrations and assay techniques used by different workers, although these could be contributing factors. PEPCK could exhibit co-operative behaviour with respect to oxaloacetate, in which case the Michaelis constant would be a function of the oxaloacetate concentration in the range examined. Utter and Kolenbrander (1972) have obtained results which seem to indicate that under certain conditions, in the presence of both manganese and magnesium, Michaelis-Menten kinetics are not observed for the chicken liver enzyme. Ballard (1970) varied the oxaloacetate concentrations over a wide range (10 μM to 3 mM) and did not observe any deviations from linearity under the conditions used. The major factor contributing to the differences in Michaelis constants is likely to be the problem caused by non-enzymic breakdown of oxaloacetate to pyruvate during the assay. It is important to prepare oxaloacetate solutions immediately before each experiment and to use true concentrations for calculations of Michaelis constants (Ballard, 1970). It is probable that in cases where high values have been obtained, oxaloacetate breakdown had occurred and the true concentrations were not used. Ballard (1970), using the Seubert-Huth assay method, made allowance for the breakdown of oxaloacetate by preparing

reaction mixtures from which PEPCK was omitted and taking the average of the initial and final oxaloacetate concentrations as the concentration available for the reaction. Meyuhas *et al.* (1971) showed differences in the Michaelis constant of the rat liver cytosol enzyme depending on whether oxaloacetate was added directly to the reaction mixture or whether it was generated *in situ* from malate; the values were reported to be 0.3 and 0.022 mM, respectively.

It is apparent from Table II that the Michaelis constants of PEPCK for ITP and GTP also vary over a considerable range. The values reported by Holten and Nordlie (1965) for the guinea pig liver cytosol and mitochondrial enzymes are 2 - 3 orders of magnitude higher than the values reported by Walsh and Chen (1971). The values obtained for the Michaelis constants of rat liver cytosol PEPCK vary over an approximately 10-fold range (Foster *et al.*, 1967; Walsh and Chen, 1971; Jomain-Baum *et al.*, 1976). For the pig liver mitochondrial enzyme the Michaelis constant for GTP is somewhat lower than for ITP (Chang *et al.*, 1966). The respective Michaelis constants reported by Holten and Nordlie (1965) and Walsh and Chen (1971) for the guinea pig liver cytosol and mitochondrial enzymes are similar for GTP and ITP. The intracellular concentration of GTP in rat liver is in the 0.1 - 0.6 mM range (Chance *et al.*, 1965; Clifford *et al.*, 1972) and the values obtained by Chang *et al.* (1966), Holten and Nordlie (1965) and Foster *et al.* (1967) for the Michaelis constants of the pig, guinea pig and rat liver enzymes for this substrate are within the same general range but perhaps a little higher. This is consistent with a regulatory role for GTP as has been suggested from studies with isolated guinea pig liver mitochondria (Garber and Ballard, 1970). The lower values reported for guinea pig and rat liver PEPCK by Walsh and Chen (1971) and Jomain-Baum *et al.* (1976), however, suggest that the enzyme would be fully saturated with respect to this nucleotide.

The reason for the discrepancies between the values reported by different workers is not clear. It is again unlikely that such wide variations can be explained solely in terms of the differences in pH, substrate concentrations and assay techniques used. Contamination of ITP or GTP by the respective nucleoside diphosphates, which in chicken and pig liver at least are competitive inhibitors (Chang *et al.*, 1966; Felicioli *et al.*, 1970), could result in high values being obtained.

Inhibitors

Studies of the effects of sulphydryl reagents have indicated that PEPCK requires free sulphydryl groups for maximum activity in catalysing the formation of phosphoenolpyruvate. It has been demonstrated that a 6-fold excess of p-hydroxymercuribenzoate produces a 98 - 100% inhibition of the chicken liver enzyme and that N-ethylmaleimide and sodium tetrathionate produce similar inhibitions (Utter and Kolenbrander, 1972). Several reagents such as 2-mercaptoethanol, reduced glutathione and dithioerythritol are able to reverse the effects of these inhibitors, with 2-mercaptoethanol being the most effective. PEPCK from pig liver is inhibited by p-chloromecuribenzoate and N-ethylmaleimide; the inhibition by p-chloro-mercuribenzoate is reversed by addition of reduced glutathione (Chang and Lane, 1966).

Studies of pig liver mitochondrial PEPCK have shown that potassium fluoride is a good inhibitor of the decarboxylation reaction, whereas sodium arsenate is ineffective (Chang and Lane, 1966).

The enzyme from chicken liver mitochondria is inhibited competitively with respect to ITP by IDP (Felicioli *et al.*, 1970). Pig liver mitochondrial PEPCK is not, however, affected by IDP but is inhibited by GDP and GMP (Chang *et al.*, 1966). Inhibition by GDP and GMP is of a mixed type having both competitive and non-competitive elements (with

respect to nucleoside triphosphate). AMP has been shown to inhibit the guinea pig liver mitochondrial enzyme competitively with respect to the phosphoryl donor (Holten and Nordlie, 1965). Bicarbonate is an effective inhibitor of the pig liver enzyme, the inhibition being competitive with respect to oxaloacetate (K_i 43 mM) (Chang *et al.*, 1966). Phosphoenolpyruvate does not inhibit pig liver PEPCK.

In the decarboxylation direction, at low concentrations of oxaloacetate, in the presence of either manganese or magnesium, the chicken liver mitochondrial, sheep liver cytosol and mitochondrial and rat liver cytosol enzymes are inhibited by malate (Ballard, 1970). At high oxaloacetate concentrations, the cytosol enzymes are inhibited only in the presence of magnesium, whereas the mitochondrial enzymes are not inhibited in the presence of either metal. The cytosol enzymes are inhibited non-competitively by malate when oxaloacetate is the variable substrate and magnesium the added cation (K_i 25 mM).

Quinolinate and 3-mercaptopicolinate, which are inhibitors of gluconeogenesis, are non-competitive (with respect to oxaloacetate) inhibitors of guinea pig liver cytosol and mitochondrial PEPCK (Robinson and Oei, 1975). The inhibitor constants for quinolinate are 0.13 and 0.17 mM for the cytosol and mitochondrial enzymes, respectively. 3-Mercaptopicolinate is a more potent inhibitor, affecting the cytosol enzyme to a greater extent than the mitochondrial form; the respective inhibitor constants are 0.005 and 0.056 mM. 3-Mercaptopicolinic acid also inhibits rat liver cytosol PEPCK non-competitively with respect to oxaloacetate or $MnGTP^{2-}$ (Jomain-Baum *et al.*, 1976). The inhibitor constants reported are within the range 0.002 - 0.009 mM.

A paradoxical effect of tryptophan on rat liver PEPCK has been observed. Gluconeogenesis is inhibited following administration of this

amino acid and the metabolic crossover point apparently occurs between oxaloacetate and phosphoenolpyruvate, suggesting that PEPCK activity is decreased (Foster *et al.*, 1966b; Ray *et al.*, 1966). Marked increases in hepatic PEPCK activity have, however, been demonstrated as a result of tryptophan treatment (Foster *et al.*, 1967). Snoke *et al.* (1971) showed that the elevated activity in tryptophan-treated rats was lowered *in vitro* by quinolinate (a metabolite of tryptophan) to the same extent as was the activity of the iron II-activated enzyme from normal rats. They also noted increased hepatic synthesis of haem following administration of tryptophan and suggested that formation of a ferrous complex of quinolinate is responsible for the effects of tryptophan. The apparent activation of PEPCK following tryptophan treatment presumably results from dissociation of the complex when the tissue homogenate is diluted for assay, leaving the enzyme in a metal-activated state.

Hydrazine administered to rats induces hypoglycaemia and *in vivo* studies have shown that, as in the case of tryptophan, it causes a metabolic crossover point between oxaloacetate and pyruvate, indicating that it inhibits PEPCK (Ray *et al.*, 1970). However, hepatic PEPCK activity increases *in vivo* following administration of hydrazine whereas *in vitro* the enzyme is inhibited (non-competitively with respect to oxaloacetate) by this compound. Ray *et al.* (1970) have suggested that the increased activity following hydrazine treatment may result from increased enzyme synthesis in response to decrease in blood glucose or stress-induced elaboration of adrenal hormones; inhibition by administered hydrazine *in vivo* is presumably not apparent because the concentration of hydrazine is decreased to a point where it is not inhibitory, by dilution of liver homogenates during the assay procedure.

Rat liver cytosol PEPCK has been shown to be inactivated by acetaldehyde and it has been suggested that this effect might contribute to inhibition of gluconeogenesis resulting from ethanol metabolism (Baxter, 1976).

Two analogues of phosphoenolpyruvate, namely phosphoenol-α-ketobutyrate and phosphoenol-α-ketovalerate have been shown to be strong inhibitors of the decarboxylation reaction catalysed by pigeon liver PEPCK (Söling *et al.*, 1971).

Foster *et al.* (1967) tested the effects of citrate, aspartate, malate, pyruvate, lactate, fumarate, succinate, α-ketoglutarate, glutamate, isocitrate, aconitate, glutamine, acetyl coenzyme A, glucose-6-phosphate, fructose-1,6-diphosphate, glucose-1-phosphate, 6-phosphogluconate, fructose-6-phosphate, α-glycerophosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, glucose, NAD^+, NADH, $NADP^+$ and NADPH on the activity of rat liver cytosol PEPCK. Of these compounds only α-glycerophosphate, which was somewhat inhibitory, had any significant effect. The activity of chicken liver PEPCK has been shown to be unaffected by coenzyme A derivatives (acetyl, propionyl, butyryl, malonyl, methylmalonyl, acetoacetyl, β-hydroxybutyryl, succinyl and palmityl), carnitine derivatives (palmityl and acetyl), AMP, CMP, GMP, TMP, citric acid cycle intermediates, amino acids, fructose-1,6-diphosphate, lactate, quinolinate and phthalate (Utter and Kolenbrander, 1972).

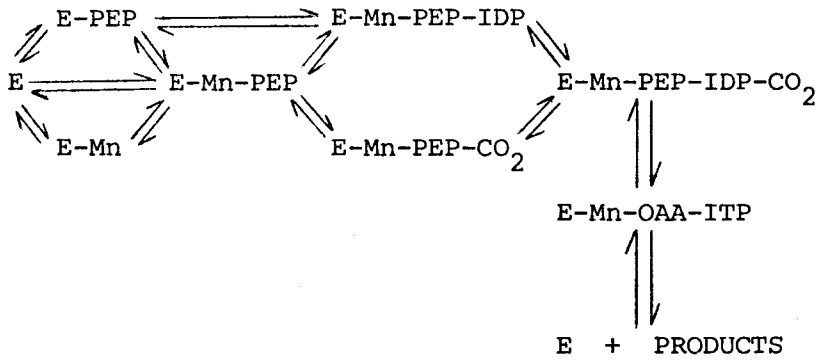
Reaction Mechanism

From studies of the kinetics of various possible exchange reactions catalysed by PEPCK from pig liver, Chang *et al.* (1966) postulated that the overall reaction might be as shown below. The nature of the events undergone by the ternary complexes was not specified, but the first reaction must be rate-limiting.

$$E + PEP + IDP \rightleftharpoons E\begin{matrix}\diagup IDP \\ \diagdown PEP\end{matrix}$$

$$E\begin{matrix}\diagup IDP \\ \diagdown PEP\end{matrix} + CO_2 \rightleftharpoons E + OAA + ITP$$

In a more comprehensive kinetic study of the mechanism of pig liver PEPCK, Miller and Lane (1968) proposed that initial steady state velocity and binding studies involving the same enzyme (Miller *et al.*, 1968) are consistent with the following mechanism.



The most significant features of this mechanism are (a) the formation of a central complex consisting of the enzyme, manganese, phosphoenolpyruvate, IDP and carbon dioxide by what the authors call a mixed ordered random addition of components; (b) phosphoenolpyruvate must bind to the enzyme before IDP or carbon dioxide to yield a kinetically active form; (c) the binding of phosphoenolpyruvate proceeds in a random manner with respect to manganese to form the ternary complex; (d) the ternary complex may bind either IDP or carbon dioxide to form a quaternary complex which then binds the third substrate.

The studies of Miller *et al.* (1968) indicated that manganese binds directly to the enzyme in an apparent 1:1 ratio and that the metal ion facilitates the binding of phosphoenolpyruvate, possibly by serving as a bridge between enzyme and substrate. It appears that two ligands of the enzyme displace two water molecules from the outer co-ordination sphere of the metal, leaving four molecules of water in the octahedral hydration shell. Phosphoenolpyruvate replaces two of the remaining four water molecules and IDP replaces one. The central decarboxylation complex is similar with two water molecules replaced by ITP and one by oxaloacetate.

The mechanism of action of PEPCK is, however, extremely complex and as far as the chemical events are concerned, the above scheme cannot be considered as more than a suggestion. In order to elucidate the mechanism fully further extensive studies are required.

ALKALINE PHOSPHATASE

General

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) catalyses the hydrolysis of almost any phosphomonoester to give phosphate and the corresponding alcohol, phenol or sugar. It is distinguished from a similar enzyme, acid phosphatase, largely by the pH dependence of its catalytic activity, which is higher in the alkaline region than at neutral or acid pH.

Alkaline phosphatase is found in bacteria and fungi but not in higher plants. There is little information concerning its distribution in invertebrate tissues but it is relatively abundant in fishes and mammals. The presence of alkaline phosphatase in mammalian tissues was first demonstrated, at the beginning of this century, by Levene and Medigreceanu (1911) and Grosser and Hüsler (1912). Extensive studies of the enzyme from tissues of a wide variety of mammalian species, including human, have since been carried out (see Fishman and Ghosh, 1967; Fernley, 1971). The major tissue sources of alkaline phosphatase are placenta, intestine, kidney, bone, liver, lung and spleen; the activities are highest in placenta and intestine. In placental tissue the enzyme is located at the surface of the trophoblastic syncytium, in intestine it is found in the microvilli, in kidney it occurs in the epithelial cells of the proximal convoluted tubules, in liver it is adjacent to the bile

canaliculi and in bone it is found in hypertrophic cartilage cells, osteoblasts and osteocytes.

At the present time, the precise function of alkaline phosphatase remains uncertain. The bone enzyme, however, is undoubtedly concerned in some way with ossification (Fleisch *et al.*, 1966). In other tissues it is perhaps significant that the enzyme is localised at the absorptive surfaces, suggesting a direct role in the transport of nutrients across the epithelial membrane (Fernley, 1971). It appears that one of the functions of alkaline phosphatase of the liver membrane is to hydrolyse phosphoryl-choline so that choline can be transported across the bile canalicular membrane (Pekarthy *et al.*, 1972).

The relationship between the various tissue alkaline phosphatases has been under consideration for many years. One of the first tissue-specific characteristics to be discovered was the observation that the bone and kidney enzymes are inhibited by bile acids whereas the intestinal form is unaffected (Bodansky, 1937). This approach was extended by Fishman *et al.* (1962, 1963) who investigated the behaviour of tissue phosphatases towards a variety of compounds. Of particular importance was the finding that L-phenylalanine is a stereospecific inhibitor of the intestinal enzyme but not of the enzymes from bone, kidney, liver or lung. Other evidence has accumulated and the various tissue isozymes have been found to differ with regard to physical, catalytic and immunological properties (Fishman and Ghosh, 1967).

Because of the clinical significance of alkaline phosphatase in connection with certain pathological conditions, notably obstructive jaundice, rickets and various bone diseases, there has been considerable interest in the human isozymes and these have been studied in detail (Moss and King, 1962; Fishman and Ghosh, 1967; Ghosh *et al.*, 1967; Ghosh and Fishman, 1968; Fishman *et al.*, 1968; Eaton and Moss, 1968; Moss,

1969a, 1971). Some of the properties of the human placental, intestinal, kidney, bone and liver isozymes are compared in Table III. It can be seen that both the placental and intestinal forms differ from the other isozymes in that they are inhibited by L-phenylalanine but not by L-homoarginine; the kidney, bone and liver isozymes resist inhibition by L-phenylalanine but are inhibited by L-homoarginine. Placental and intestinal phosphatases show differences with respect to pH optima, Michaelis constants for phenyl phosphate heat stability, electrophoretic mobility and effect of neuraminidase on electrophoretic mobility. The differences between the kidney, bone, liver and other non-placental, non-intestinal isozymes are, however, much less clear-cut. The kidney isozyme can be distinguished from the liver and bone isozymes by means of its electrophoretic mobility. There are small, but reproducible, differences between the Michaelis constants of bone and liver alkaline phosphatases for β-naphthyl phosphate. The liver isozyme is more resistant to inactivation by heat, urea and acid pH than bone phosphatase and these differences are maintained in mixtures of the two enzymes.

Investigations of the molecular variations of alkaline phosphatase within a single tissue have been carried out (Moss and King, 1962; Moss, 1969a). When phosphatase-containing extracts of human tissues are subjected to starch gel electrophoresis at pH 8.6, the main zones of activity show varying degrees of heterogeneity, ranging from a fairly compact band in liver extracts to a very diffuse region in extracts of kidney. Additional slowly-migrating, minor zones are present in all extracts. Studies of the properties (e.g. Michaelis constants, pH optima, heat stability) of these zones, after recovery from starch gels, have indicated that within a single tissue, the properties of the main enzyme zone are reflected in the minor bands, and that, within the more diffuse main zones (such as those of intestinal and kidney phosphatases) there is apparently no variation in the properties. It therefore appears that there is only a single alkaline phosphatase within each tissue and that the observed heterogeneity is a

TABLE III

COMPARISON OF HUMAN ALKALINE PHOSPHATASE ISOZYMES

PROPERTY	CONDITIONS	PLACENTA	INTESTINE	KIDNEY	BONE	LIVER	REFERENCES
Optimal pH	18 mM phenyl phosphate 50 mM carbonate-bicarbonate buffer	10.6	9.9				Fishman and Ghosh (1967)
	5 mM β-naphthyl phosphate 100 mM carbonate-bicarbonate buffer		10.1	10.2	10.1	10.1	Moss and King (1962)
Michaelis constants (mM)							
Phenyl phosphate	50 mM carbonate-bicarbonate buffer pH 9.3	0.37	0.16				Fishman and Ghosh (1967)
β-Naphthyl phosphate	100 mM carbonate-bicarbonate buffer pH 10.1		0.09	0.104	0.103	0.067	Moss and King (1962)
Inhibition (%)							
L-Phenylalanine 5 mM	18 mM phenyl phosphate 50 mM carbonate-bicarbonate buffer pH 9.3	79	77	14.3	10.3	7.7	Fishman and Ghosh (1967)
L-Homoarginine 10 mM	18 mM phenyl phosphate 50 mM carbonate-bicarbonate buffer pH 9.8	0.5	<10		>80	73.1	Fishman and Sie (1970) Lin and Fishman (1972)
Heat Stability							
Time for half-inactivation at 55° (min)	5 mM β-naphthyl phosphate 100 mM carbonate-bicarbonate buffer pH 7.0	Completely stable	15	12	8	18	Moss and King (1962) Neale *et al.* (1965)
Urea concentration for irreversible inactivation (M)		8	6-7	<3	<3	3	Moss (1969a)
Electrophoretic mobility		Slightly faster than intestinal isozyme	Slightly slower than placental isozyme	Similar to intestinal isozyme	Similar to placental isozyme	Similar to placental isozyme	Fishman and Ghosh (1967)
Effect of neuraminidase on electrophoretic mobility		Retarded	Not retarded	Retarded	Retarded	Retarded	Moss (1969a)

consequence of modification of this single enzyme in some way. It has been suggested that the minor zones may represent aggregates of the enzyme with itself or with non-enzymic proteins, be degradation products or be due to a variable content of sialic acid residues (Moss, 1969a).

As is the case for most enzymes, the properties of alkaline phosphatase in a particular tissue often differ somewhat from one species to another. This is illustrated in Table IV, in which the properties of various placental isozymes, determined under similar conditions, are compared. Small, but obvious, differences are observed between the rat, mouse, guinea pig, rabbit, monkey and baboon isozymes; the optimal concentrations of phenyl phosphate, pH optima and Michaelis constants for phenyl phosphate range respectively from 35 - 55 mM, pH 10.1 - 10.6 and 6.1 - 16.2 mM. The hamster placental isozyme is completely insensitive to L-phenylalanine while the enzyme from other non-human species shows only 7 - 12% inhibition. Monkey and baboon placental phosphatases are somewhat less thermolabile than the rat, mouse, guinea pig, hamster or rabbit isozymes. The optimal phenyl phosphate concentration and Michaelis constant for the phenyl phosphate are slightly higher for the human placental isozyme than for the enzyme from other species. Human placental alkaline phosphatase differs markedly from the other enzymes with respect to L-phenylalanine inhibition and heat stability.

Enzyme assays

Many methods for the determination of alkaline phosphatase activity involve colorimetric measurement of phosphate released during the reaction (Itaya and Ui, 1966; Van Belle, 1970). In practice, however, it is often more convenient to measure the release of a phenol from an aryl phosphomonoester. Standard methods, in which the phenol released

TABLE IV

COMPARISON OF PLACENTAL ALKALINE PHOSPHATASE ISOZYMES OF VARIOUS SPECIES

PROPERTY	CONDITIONS	RAT	MOUSE	GUINEA PIG	HAMSTER	RABBIT	AFRICAN GREEN MONKEY	RHESUS MONKEY	BABOON	HUMAN
Optimal substrate concentration Phenyl phosphate (mM)	50 mM carbonate-bicarbonate buffer Optimal pH for each isozyme	35	50	55	45	35	40	40	35	72
Optimal pH	Phenyl phosphate as substrate (used at optimal concentration) 50 mM carbonate-bicarbonate buffer	10.5	10.2-10.3	10.2	10.2	10.1	10.4	10.5-10.6	10.3	10.7
Michaelis constants Phenyl phosphate (mM)	50 mM carbonate-bicarbonate buffer Optimal pH for each isozyme	11.9	9.5	12.4	10.2	6.1	12.3	8.7	16.2	18
Inhibition (%) L-Phenylalanine 5 mM	18 mM phenyl phosphate 25 mM carbonate-bicarbonate buffer pH 9.8	11	8	7	0	11	12	10	11	80
Heat inactivation (%) 16 minutes at 55°	18 mM phenyl phosphate 25 mM carbonate-bicarbonate buffer pH 9.8	92	83	95	100	80	67	61	66	1-2

References:- Rat isozyme: Manning *et al.* (1969a).

Mouse, guinea pig, hamster and rabbit isozymes: Manning *et al.* (1970).

African green monkey, rhesus monkey and baboon isozymes: Manning *et al.* (1969b).

Human isozyme: Fishman and Ghosh (1967), Ghosh *et al.* (1967), Ghosh and Fishman (1968) and Fishman *et al.* (1968).

is measured spectrophotometrically, employ phenyl phosphate (Kind and King, 1954), p-nitrophenyl phosphate (Bessey *et al.*, 1946; Bowers and McComb, 1966), phenolphthalein monophosphate (Babson *et al.*, 1966) and thymolphthalein monophosphate (Coleman, 1966). A number of more sensitive fluorimetric assays have also been developed, using the fluorogenic substrates β-naphthyl phosphate (Moss *et al.*, 1961; Greenberg 1962), 4-methylumbelliferyl phosphate (Fernley and Walker, 1965) and 3-O-methylfluorescein phosphate (Hill *et al.*, 1968).

Enzyme purification

Alkaline phosphatase has been purified from many tissues. The most highly purified preparations have been obtained from calf intestinal mucosa (Morton, 1954; Portmann, 1957), horse and pig kidney (Mathies, 1958; Binkley, 1961), bovine liver (Engström, 1964), human placenta (Georgatsos, 1967; Sussman *et al.*, 1968; Harkness, 1968a; Ghosh and Fishman, 1968) and human liver (Trépanier *et al.*, 1976). The enzyme is normally firmly attached to the lipoprotein membranes but can be solubilised either by autolysis or by treatment with n-butanol. Crude extracts are usually subjected to a series of fractionations with ammonium sulphate, acetone or ethanol. Further purification has been achieved by means of ion-exchange chromatography, gel filtration and more recently affinity chromatography.

Physical properties

The molecular weights of the enzyme from calf intestinal mucosa, bovine liver, human placenta and human liver have been reported to be 100,000 (Engström, 1961a), 150,000 (Engström, 1964), 125,000 (Harkness, 1968a) and 146,000 (Trépanier *et al.*, 1976), respectively.

The human placental enzyme is thought to be a dimer of equal weight subunits which are not necessarily identical; genetic studies have indicated that there are three types of subunit which can combine to give six electrophoretically distinguishable variants (Robson and Harris, 1967; Gottlieb and Sussman, 1968). Higher molecular weight forms of placental alkaline phosphatase have been observed (Robson and Harris, 1967; Ghosh and Fishman, 1968) and at pH values greater than 10.5 reversible dissociation into monomers has been shown to occur (Sussman and Gottlieb, 1969).

Titration curves for calf intestinal phosphatase indicate an isoelectric point of 5.7 (Lazdunski *et al.*, 1965). By means of polyacrylamide gel isoelectric focusing, Smith *et al.* (1971) found the isoelectric point of the human liver enzyme to be in the region pH 5.0 - 5.3, whereas using isoelectric focusing in a sucrose density gradient, Latner *et al.* (1970) and Trépanier *et al.* (1976) obtained values 3.9 and 4.0, respectively for this enzyme.

The metal-ion status of alkaline phosphatase is now reasonably well resolved (Mathies, 1958; Engström, 1961a; Harkness, 1968a). It is a zinc metalloenzyme with 2 - 3 g-atom of zinc per mole of enzyme. The metal is essential for catalytic activity and possibly also for the maintenance of native enzyme structure. Amino acid compositions of placental phosphatase have been published; the relative percentages of amino acid residues on a molar basis are 21% acidic, 14% basic, 15% hydrophilic and 50% non-polar (Harkness, 1968a; Sussman and Gottlieb, 1969).

Kinetic studies

Substrate specificity

It has long been recognised that alkaline phosphatase

hydrolyses a wide variety of orthophosphate derivatives including hexose phosphates, glycerophosphates, phenyl phosphate, nitrophenyl phosphate, methylphenyl phosphates, naphthyl phosphates and pyridoxal phosphate. There has, however, been some confusion as to whether the enzyme also catalyses the hydrolysis of inorganic and organic pyrophosphate derivatives. Morton (1955) defined the substrate specificity of calf intestinal phosphatase; as well as monoesters, phosphocreatine was found to be a substrate but pyrophosphate derivatives such as PP_i, ADP, ATP, diesters (such as diphenyl phosphate) and triesters (such as trimethyl phosphate) were not hydrolysed. Portmann (1957) confirmed that PP_i, ADP and diphenyl phosphate were not substrates. It was subsequently reported that phosphatases from several mammalian tissues had appreciable PP_i-ase activity (Cox and Griffin, 1965). This was confirmed and extended to include ATPase and fluorophosphatase activities (Moss *et al.*, 1967; Fernley and Walker, 1967). The studies of Fernley and Walker (1967) indicated that the main reason for the confusion with regard to pyrophosphatase activity, results from the inclusion of magnesium in the assay; this stimulates monoesterase activity but almost completely inhibits PP_i-ase activity.

Harkness (1968b) has carried out a detailed study of the substrate specificity of human placental alkaline phosphatase. The enzyme hydrolyses glucose-1-phosphate, α-glycerophosphate, β-glycerophosphate, 2-phosphoglycerate, 3-phosphoglycerate, phosphoserine, phosphoethanolamine, p-nitrophenyl phosphate, adenosine and uridine mono-, di- and triphosphates and inorganic pyrophosphate. Compounds not hydrolysed include trimetaphosphate, bis-p-nitrophenyl phosphate, phosphocreatine and aminophosphonates.

pH Optima

There are numerous reports in the literature of changes in pH

optima in the presence of different substrates; as an example, the pH optimum of the human intestinal enzyme is 8.0 in the presence of β-glycerophosphate and 9.8 in the presence of phenyl phosphate (Fishman and Ghosh, 1967).

Increases in pH optima with increasing substrate concentration have been reported for various tissue phosphatases. Examples of this type of behaviour are found with calf intestinal phosphatase and phenyl phosphate (Morton, 1957), chicken or rat intestinal phosphatases and β-glycerophosphate (Motzok and Wynne, 1950; Ross *et al.*, 1951), human bone phosphatase and p-nitrophenyl phosphate (Eaton and Moss, 1968) and human intestinal, bone and liver phosphatases and β-naphthyl phosphate (Moss *et al.*, 1961). A linear relationship between the logarithm of the substrate concentration and the pH optimum has been observed for the enzyme from several species (Ross *et al.*, 1951; Motzok and Branion, 1959; Motzok, 1963).

Ahmed and King (1960) have demonstrated shifts in the pH-activity curves on changing from one buffer system to another. For human placental alkaline phosphatase and phenyl phosphate as substrate, the pH optimum is higher with ethanolamine (10.2) than with veronal (9.9) or glycine (9.6) buffers; with β-glycerophosphate as substrate, the pH optimum in the presence of veronal buffer (9.9) is slightly higher than in the presence of ethanolamine (9.7) or glycine (9.6). The effects of changing the buffer may possibly be associated with changes in ionic strength. Furthermore, addition of metal ions, which may also contribute to the total ionic strength, causes shifts in pH optima (Motzok and Branion, 1959)

Michaelis constants

The Michaelis constants of alkaline phosphatase vary according to the

substrate used. The Michaelis constants of human tissue phosphatases have been determined for many different substrates (Fishman and Ghosh, 1967). The values reported for human placental alkaline phosphatase (in the presence of 0.1 M glycine buffer at pH 10.5) are 3.0, 4.4, 5.7, 4.0, 4.8, 1.4, 2.9 and 0.8 for AMP, ADP, ATP, PP_i, glucose-1-phosphate, β-glycerophosphate, phosphoethanolamine and p-nitrophenyl phosphate, respectively (Harkness, 1968b). The Michaelis constants of the human liver and intestinal enzymes for thymolphthalein monophosphate are 10 - 20 times lower than those for O-carboxyphenyl phosphate, determined under similar conditions (Moss, 1971). Differences between the Michaelis constants of the human intestinal and kidney enzymes for phenyl phosphate and β-glycerophosphate and the Michaelis constants of the brain enzyme for pyridoxal phosphate, AMP and phenyl phosphate have been reported (Ahmed and King, 1960; Saraswathi and Bachhawat, 1963).

In general it is found that the Michaelis constants for a particular substrate increase with increasing pH. For example, Fishman and Ghosh (1967) and Fishman *et al.* (1968) have determined the Michaelis constants of human placental alkaline phosphatase for phenyl phosphate, in the presence of carbonate-bicarbonate buffer, at several pH values ranging from 8.9 to 10.5 and have shown them to increase from 0.32 mM, at the lower pH value, to 17 mM at the higher pH. Using the same substrate, under similar conditions, Fishman *et al.* (1968) reported increases in the Michaelis constants of the human intestinal enzyme from 0.06 mM at pH 9.2 to 17 mM at pH 10.7. The Michaelis constants of the human intestinal, bone and liver phosphatases for p-nitrophenyl phosphate are 40 - 70-fold higher at pH 10.5 than at pH 7.0 (Eaton and Moss, 1968).

It is usually found that plots of pK_m against pH have linear regions, often with non-integral slopes. Such behaviour has been observed

for calf intestinal phosphatase and p-nitrophenyl phosphate or 4-methylumbelliferyl phosphate (Lazdunski and Ouellet, 1961; Fernley and Walker, 1965), rat intestinal and human placental phosphatases and phenyl phosphate (Fishman and Ghosh, 1967) and human intestinal, bone and liver phosphatases and p-nitrophenyl phosphate (Eaton and Moss, 1968).

The fact that, for a given substrate, the Michaelis constant increases with increasing pH while maximum velocity approaches a limiting value (Lazdunski and Ouellet, 1961; Fernley and Walker, 1965) is sufficient to explain, at least qualitatively, the change in pH optimum with substrate concentration. Also changing the buffer or ionic strength may be expected, by virtue of the sensitivity of the Michaelis constant and maximum velocity to the environment of the enzyme, to produce shifts in the pH dependencies of these parameters and hence in the pH-activity curves.

Inhibitors

The amino group reagents, ketene, nitrous acid, formaldehyde and phenyl isocyanate have been shown to inactivate bovine liver and kidney phosphatases (Anagnostopoulos, 1950). Rat intestinal phosphatase is inactivated by acetic anhydride but there is little change in the kinetic parameters (Fishman and Ghosh, 1967). Similarly, acetylation of human tissue phosphatases with acetic anhydride gives low yields of active enzyme with altered electrophoretic mobilities, but with little change in Michaelis constant or pH optimum (Moss, 1970). Cyanate inhibits the human and pig kidney enzymes, the effects being both time and substrate dependent; at high cyanate concentration a product with increased Michaelis constant is produced (Carey and Butterworth, 1969). Although

these studies have not been related to active site determinations, it is, however, clear that some of the amino groups of alkaline phosphatase can be modified without the binding of substrate being affected.

p-Hydroxymercuribenzoate, N-ethylmaleimide, iodoacetamide and iodosobenzoate have been found to inhibit intestinal phosphatases, but high concentrations are required (Lazdunski and Ouellet, 1962; Fishman and Ghosh, 1967). It is therefore doubtful whether alkaline phosphatase contains essential sulphydryl groups or indeed possesses any free sulphydryl groups.

Alkaline phosphatases are inhibited by excess substrate and it has been found that this inhibition generally determines the shape of the acid limb of the pH-activity curves at high substrate concentration (Fernley, 1971). It is possible that additional substrate molecules may combine at the same site, at identical but different sites or perhaps with a phosphoryl-enzyme intermediate to form inactive complexes.

Phosphate is an effective competitive inhibitor of alkaline phosphatases, with an affinity comparable to that for good substrates (Lazdunski and Ouellet, 1962; Fernley and Walker, 1967). Consequently, the kinetics of hydrolysis are often approximately first order whatever the substrate concentration and for this reason initial rate measurements should be limited to 10% hydrolysis. Another powerful competitive inhibitor is arsenate (Morton, 1955; Harkness, 1968b). Phosphonates have been reported as weak competitive inhibitors of human intestinal phosphatase (Kochman *et al.*, 1964).

The amino-acids L-phenylalanine, L-tryptophan and L-homoarginine are organ-specific inhibitors of alkaline phosphatase. L-Phenylalanine is a strong inhibitor of the bovine, rat, rabbit, pigeon and human

intestinal enzymes and the human placental enzyme; the mouse, rat, rabbit, guinea pig, hamster, monkey and baboon placental enzymes and the rat and human bone, liver, kidney and lung enzymes are only weakly inhibited (Fishman *et al.*, 1962, 1963; Fishman and Ghosh, 1967; Brunel and Cathala, 1972). L-Tryptophan is also a strong inhibitor of the human placental and intestinal alkaline phosphatases, whereas L-homoarginine is a potent inhibitor of the rat and human liver and bone enzymes but not of the enzyme from other tissues (Fishman and Sie, 1970; Lin and Fishman, 1972; Brunel and Cathala, 1972).

Inhibition by L-phenylalanine and L-tryptophan is known to be stereospecific, the D-isomers being without effect; this is probably also the case for L-homoarginine (Fishman *et al.*, 1963; Lin and Fishman, 1972). The mechanism of inhibition for all three compounds has been shown to be uncompetitive (Ghosh and Fishman, 1966, 1968; Fishman and Ghosh, 1967; Lin *et al.*, 1971; Lin and Fishman, 1972). From studies of L-phenylalanine inhibition, it was suggested by Fishman and Ghosh (1967) that an inactive enzyme-substrate-inhibitor complex was formed by stereospecific combination of the amino acid with the enzyme-substrate complex. The studies of Fernley and Walker (1970), however, indicated that combination with L-phenylalanine has little effect on the reactivity of the enzyme-substrate complex, but prevents the breakdown of a phosphoryl-enzyme intermediate formed later in the hydrolysis sequence, following release of the non-phosphate product.

Two other amino acids, L-arginine and L-lysine, have also been shown to be stereospecific inhibitors of human alkaline phosphatase, affecting the liver enzyme to a greater extent than the placental form (Fishman and Sie, 1970). It is possible that these are also uncompetitive inhibitors, but this has not yet been confirmed.

Another organ-specific inhibitor of alkaline phosphatase is imidazole (Brunel and Cathala, 1972). This compound inhibits the rat liver, bone, lung and spleen and rat and bovine kidney and brain enzymes but has little effect on the enzymes from rat, bovine, rabbit or pigeon intestine or human placenta. Studies of the bovine brain enzyme have shown that the inhibition is dependent on pH, greatly dependent on substrate and inhibitor concentrations and of an uncompetitive type.

Inhibition of alkaline phosphatase by cysteine, cysteine analogues and histidine is different from that of the other amino acids mentioned above and imidazole. Cysteine is a very strong inhibitor of the human kidney and rat bone, liver and intestinal isozymes, the extent of inhibition being similar in each case (Agus *et al.*, 1966). Analogues of cysteine with free amino and sulphydryl groups (e.g. penicillamine, homocysteine, cysteine ethyl ester) are also effective inhibitors of the human kidney enzyme, whereas analogues with blocked amino or sulphydryl groups and compounds lacking these groups (e.g. N-acetyl-L-cysteine, mercaptoacetic acid, β-mercapto-propionic acid) are comparatively poor inhibitors. Inhibition of the human kidney enzyme by cysteine is pH dependent, non-competitive and non-stereospecific. The inhibition is reversed by addition of zinc but not by calcium or magnesium. Agus *et al.* (1966) have suggested that inhibition of alkaline phosphatase by cysteine is a result of chelation *in situ* with the zinc atom at the active site. Histidine inhibits the enzyme from tissues of several species, although its action is slightly stronger on those isozymes (such as liver, kidney and bone) which are least sensitive to L-phenylalanine inhibition (Brunel and Cathala, 1972). The inhibition is non-competitive or mixed reaching a maximum at pH 10.0. D- and L-histidine produce the same inhibition; there is no stereospecific effect.

EDTA has been shown to have an inhibitory effect on alkaline phosphatase. Hofstee (1955) showed that dialysis of calf intestinal phosphatase against EDTA produced an inactive enzyme; addition of zinc led to reactivation while addition of magnesium did not. Morton (1957) confirmed that the enzyme was inactivated by EDTA and obtained partial recovery with magnesium or manganese. Mathies (1958) found that dialysis of pig kidney phosphatase against EDTA or Mg-EDTA resulted in considerable loss of activity and a parallel reduction in the enzyme zinc content. A similar correlation between activity and zinc content has been found for calf intestinal phosphatase, although in this instance activity is not restored by addition of zinc (Engström, 1961a).

The kinetics of EDTA inhibition of human tissue phosphatases have been shown to be complex being both time and substrate dependent; however, it is clear that the presence of substrate offers limited protection against inactivation (Conyers *et al.*, 1967; Harkness, 1968b). Three different effects of EDTA on bone, intestinal and placental isozymes have been observed by Conyers *et al.* (1967). Low concentrations of EDTA (10^{-5} - 10^{-3} M) in the assay system produce a loss of activity which is the same irrespective of the EDTA concentration. It has been suggested that the inhibition could be independent of the EDTA concentration as a result of the presence of an activator, possibly a metal, in the enzyme preparations or reagents used. At concentrations of EDTA above 10^{-3} M in the assay system, bone and intestinal phosphatases display an increasing loss in activity with increasing EDTA concentrations, whereas the placental enzyme shows a progressive gain in activity. The effect on the bone and intestinal enzymes may be due either to chelation of EDTA with metal ions or to a direct effect of EDTA on the enzyme molecule; the activating effect may be confined to the placental enzyme because of its

very stable structure. The third effect noted by Conyers *et al.* (1967) was that preincubation of the three phosphatases with EDTA produces a time-dependent inactivation which is not reversible by dilution. They explained this in terms of the postulate that EDTA removes a functionally essential metal (presumably zinc), either by binding the metal *in situ* and slowly inducing conformational changes in the protein structure or by immediately removing the metal from the protein leaving an unstable enzyme molecule, which then undergoes denaturation. The different rates of inactivation displayed by the various isozymes have been attributed to slight structural differences. Harkness (1968b), using crystalline placental phosphatase, has also demonstrated a time-dependent inactivation effect of EDTA; full activity can, however, be restored by addition of zinc whereas addition of magnesium produces only partial reactivation. The results obtained by Harkness (1968b) indicate that EDTA is initially bound to the enzyme and that only prolonged incubation leads to dissociation of zinc.

Other compounds which inhibit various tissue phosphatases, presumably by virtue of their zinc-chelating ability, include cyanide, O-phenanthroline, 8-hydroxyquinoline and α,α'-dipyridyl (Cloetens, 1941; Fishman and Ghosh, 1967; Harkness, 1968b).

Effect of metal ions

A perplexing aspect of alkaline phosphatase behaviour is the variety of different effects which have been demonstrated on addition of divalent metal ions. Activation of various tissue phosphatases is observed with magnesium, manganese and cobalt; other ions, notably beryllium and zinc are inhibitory, while calcium, nickel and cadmium appear to have little effect (Morton, 1955; Hofstee, 1955; Portmann, 1957; Ahmed and King, 1960; Harkness, 1968a; Fernley, 1971). Inhibition of the calf intestinal and human placental enzymes by zinc is not observed

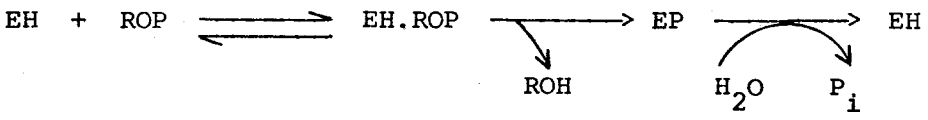
in the presence of glycine (Hofstee, 1955; Harkness, 1968a). Inhibition of the calf intestinal enzyme by zinc or beryllium can be reversed by addition of magnesium, although the effect appears to be rather complex (Morton, 1957). Moss (1969b) has reported that inhibition of human tissue phosphatases by zinc is non-competitive or mixed.

The kinetics of magnesium activation have been extensively investigated, though not usually in detail. To give an example, the activation of the calf intestinal enzyme approximately follows a titration curve with a metal ion-enzyme dissociation constant of about 0.5 mM (Morton, 1957). At high magnesium concentrations there is a slight drop in activity which may be a non-specific ionic strength effect. An important feature of the activation is that magnesium does not affect the affinity for substrate while the affinity for magnesium has a different pH dependence from that for substrate (Fernley, 1971). Studies with the phenylalanine-inhibited human placental enzyme have indicated that magnesium may act by increasing the number of active sites rather than by enhancing a particular rate (Fernley and Walker, 1970). It is possible that magnesium brings additional sites on the same molecule into action, although this cannot be the only mechanism because of the very large increases sometimes observed. Another possibility is that magnesium facilitates a redistribution of zinc, either by restoring it to an essential site or perhaps by removing it from an inhibitory site.

Reaction mechanism

Extensive investigations carried out by Engström (1961b, 1961c, 1964), using isotopic labelling techniques, have provided strong evidence that a phosphoryl enzyme is an intermediate in the hydrolysis of phosphomonoesters. These studies also showed that phosphorylation takes place

at a serine group on the protein molecule and that the presence of zinc is essential for the process. The evidence available at present is consistent with the sequence of steps shown below, in which ROP represents the substrate, EH and EP are free and phosphoryl enzyme, respectively, and EH.ROP is the Michaelis complex (Fernley, 1971).



It appears likely that the ionising group involved in the binding of substrate to mammalian alkaline phosphatases is in fact zinc hydrate (Fernley, 1971). It is also possible that two zinc groups may be associated with each active site, since plots of pK_m against pH can have negative slopes greater than unity, the difference not being accountable for by changes in maximum velocity (Lazdunski and Ouellet, 1961; Fernley and Walker, 1965).

There is some evidence in support of the idea that the Michaelis complex and the phosphorylated enzyme intermediate may have different conformations (Fernley, 1971). It is possible that the initial Michaelis complex is catalytically inert and that an isomerisation step is required to generate an active site or, alternatively, conformational change and phosphoryl transfer could occur simultaneously.

The serine group which becomes phosphorylated does not appear to possess any marked nucleophilic activity, nor is there any evidence that a histidine group participates as a general acid-base catalyst (Fernley, 1971).

AIM OF THIS STUDY

The primary aim of this work was to obtain a deeper insight into the metabolism of primate liver and the changes occurring in hepatocellular carcinoma.

Studies of the metabolic processes and associated enzyme changes in tumour tissues are important as a means of obtaining a better understanding of the effects of the malignant transformation at the molecular level. There is a high incidence of human primary hepatoma amongst the Bantu population of Southern Africa (Prates, 1961) and a series of investigations of the biochemical differences between the hepatoma and normal liver tissue has been undertaken in this laboratory. This tumour, which appears to be of an aetiology distinct from that of the subjects of Boxer and Shonk (1966) and Yeung *et al.* (1973), is fast-growing and rapidly fatal.

The earlier work carried out by others here, showed that the activities of several enzymes of carbohydrate metabolism were changed in the human hepatoma relative to those of normal or adjacent, apparently uninvolved 'host' liver (Balinsky *et al.*, 1973a). The purpose of the present work was to extend the investigation to include the key gluconeogenic enzymes, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase and the key enzyme of glycogen synthesis, glycogen synthetase.

It was also shown previously that hexokinase and pyruvate kinase isozyme patterns in the human hepatoma differed from those of normal or host liver; occasional differences were noted for lactate dehydrogenase (Balinsky *et al.*, 1973a). These studies have now been extended and the isozyme patterns of two other enzymes, namely phosphoglucomutase and glucose-6-phosphate dehydrogenase have also been examined.

Since foetal liver, unlike adult liver, is a growing, dividing tissue, comparison of its enzyme activities and isozyme patterns with those of hepatoma was considered of interest. In addition, the availability of a transplantable rat hepatoma and cell lines derived from human and rat hepatomas permitted comparison with these systems. Isozyme studies of other human tumour tissues (oesophageal cancer, meningioma and mesothelioma) and cell cultures (oesophageal cancer and HeLa) have also been carried out.

Another aspect of this work concerns a detailed kinetic study of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase. Species differences with respect to subcellular distribution, regulation and kinetic properties of this enzyme have been noted (Utter and Kolenbrander, 1972). The enzyme has not, however, been isolated from any primate tissue and characterised. The aim of this investigation was therefore to purify phosphoenolpyruvate carboxykinase from monkey liver and to examined some of the properties of mitochondrial and cytosol forms, in order to determine the extent to which these enzymes differ from each other and from the enzymes from non-primate species. The molecular weights of the monkey liver enzymes were determined and studies of nucleoside phosphate specificity, pH optima, effects of divalent metal ions, Michaelis constants for the various substrates and effects of possible modulators, in the directions of oxaloacetate or phosphoenolpyruvate formation, were carried out.

This thesis also presents a kinetic study of the alkaline phosphatase isozyme purified from the livers of baboons which had undergone auxiliary transplant operations and hence had elevated levels of the enzyme. Since human placental alkaline phosphatase has been shown to be more sensitive to inhibition by L-phenylalanine and more thermostable than the placental enzyme from baboon or monkey, it was of interest to examine the effects of amino acids and heat on the baboon liver enzyme, and to

compare the results with those reported by others for the human liver enzyme and for the enzymes from other primate tissues. The Michaelis constants of the baboon liver enzyme for p-nitrophenyl phosphate and phenolphthalein monophosphate were also determined and the effects of EDTA and divalent metal ions on this enzyme have been examined.

CHAPTER I

ACTIVITIES OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE IN LIVERS, HEPATOMAS AND HEPATOMA CELL CULTURES

SUMMARY

1. The activities of three key gluconeogenic enzymes, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) and a key enzyme of glycogen metabolism, glycogen synthetase (GS) were determined in rat and human livers, hepatomas and hepatoma cell cultures.

2. The levels of PC, PEPCK and G6Pase were significantly decreased in rat hepatomas compared with host livers, indicating a probable reduction in the rate of gluconeogenesis in the tumour. This reduction was also evident in human hepatomas in which PC and PEPCK levels were lower than the corresponding host levels. G6Pase activities were generally lower in human hepatomas, but occasionally a higher level than the host level was obtained.

3. Glycogen synthetase activity was significantly lower in both rat and human hepatomas when compared with the corresponding host levels, perhaps indicating reduced glycogen synthesis in the tumours. In rat host livers and hepatomas, the b (G6P-dependent) form of GS was predominant, whereas in human host livers and hepatomas the relative proportions of the a and b forms were extremely variable.

4. In rat hepatoma cells, PEPCK and G6Pase levels were similar to those in rat hepatomas, whereas PC levels were lower. PC, PEPCK and G6Pase levels were lower in human hepatoma cells than in

human hepatomas obtained at autopsy. The low levels of these enzymes suggest a low rate of gluconeogenesis in the cells, as in the tumours from which they were derived. In both rat and human hepatoma cells GS a levels were similar to those in the respective hepatomas, whereas GS b levels were higher and could perhaps correlate with the presence of glycogen particles in the cells.

5. All four enzymes were detected in human foetal livers. PC, PEPCK and G6Pase levels were, in general, lower than in adult livers, whereas levels of GS varied over a wide range and included values higher than those of adult livers. There did not appear to be any definite correlation of enzyme activities with foetal age except possibly for GS.

METHODS

Chemicals

Substrates and cofactors

Monosodium phosphoenolpyruvate (PEP), disodium ATP, disodium NADH and malate dehydrogenase (MDH) were obtained from Biochemica Boehringer, Mannheim, Germany; disodium IDP, trilithium S-acetyl coenzyme A (AcCoA), sodium uridine diphosphoglucose (UDPG) and glycogen (type III from rabbit liver) from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; potassium pyruvate from Calbiochem, Los Angeles, California, U.S.A. and disodium glucose-6-phosphate (G6P) from British Drug Houses, Poole, England. Sodium bicarbonate-^{14}C and uridine diphospho(D-glucose-6-^{3}H) (ammonium salt) were from the Radiochemical Centre, Amersham, England.

Other chemicals

Dithiothreitol (DTT) was obtained from Calbiochem; glutathione (reduced form) from Sigma; Folin Ciocalteu's reagent from E. Merck, Darmstadt, Germany and bovine plasma albumin from Armour Pharmaceutical Co., Eastbourne, England. Instagel scintillation fluid was from Packard Instrument Co., Downers Grove, Illinois, U.S.A. Other standard chemicals were obtained from British Drug Houses, Poole, England, E. Merck, Darmstadt, Germany and Hopkin and Williams, Chadwell Heath, England and were of analytical grade.

Tissues and Cell Cultures

Hepatoma-bearing rats were provided by Drs. C.F. Albrecht and L.D. Nourse. The rats (albino Wistar) were bred at the Council for

Scientific and Industrial Research, Pretoria, South Africa. Hepatomas were induced by means of the carcinogen 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB) and transplanted into the hind legs as described by Albrecht *et al.* (1972). Normal livers were obtained from albino Wistar rats bred at The South African Institute for Medical Research, Johannesburg, South Africa. The rats were killed by cervical dislocation, the livers removed and hepatomas dissected out; any necrotic tissue was discarded. Normal and host livers and hepatomas were kept on ice and assayed immediately.

Human livers were obtained at autopsy and in one case during laparotomy, from patients in whom primary hepatocellular carcinoma had been confirmed histologically. The patients were Bantu-speaking Negro males originating from Mocambique and working as mine labourers in South Africa. The cancerous regions of the livers were dissected out from the regions which appeared to be morphologically normal (host liver). In addition, hepatoma and host liver were obtained during surgery from a Bantu-speaking Negro female of South African origin. Hepatoma tissue was frequently necrotic and only that in which there was less than 15% necrosis (as determined histologically by the Histopathology Department of The South African Institute for Medical Research) was assayed. Normal human livers were obtained at autopsy from patients known not to have had any liver disease. Human foetal livers were obtained from spontaneously or therapeutically aborted foetuses at varying stages of gestation and were of Caucasian origin. Autopsy tissues were assayed within a maximum of six hours after death and foetal tissues as soon as possible after abortion, usually within 2 - 6 hours. After removal from the body, the tissues were kept on ice until assayed. In a few cases, if the enzyme was known to be stable, the tissue was frozen and assayed later.

A rat hepatoma cell line was derived from the above-mentioned

3'-MeDAB-induced hepatoma by Dr. L.D. Nourse and grown at 37° in Eagle's Minimum Essential Medium with Hank's base and L-glutamine without sodium bicarbonate and supplemented with 0.28 M ascorbic acid, 0.1 mM serine, 0.1 mM glycine, 9 mM sodium bicarbonate, penicillin (50 units/ml), streptomycin (50 units/ml) and 5% foetal bovine serum (Albrecht *et al.* 1975). These cells were subsequently grown by Professor O.W. Prozesky under the conditions described below for human hepatoma cells.

A human hepatoma cell line, established from the tumour of a patient in whom primary liver cancer had been confirmed histologically, was provided by Professor O.W. Prozesky. The human hepatoma cells were grown at 36° with 5% carbon dioxide in a mixture (50/50) of Eagle's Basal Medium and Dulbecco's modified Eagle's medium supplemented with penicillin G (100 units/ml), streptomycin (0.1 mg/ml) and 10% foetal calf serum (Prozesky *et al.* 1973).

Unless otherwise stated, the cells were obtained in serum-free medium after trypsinisation. They were centrifuged at 50 g, at room temperature, for two minutes, and washed twice with 0.9% saline. Extracts were prepared immediately.

Preparation of extracts and assay procedures

Assays were performed in duplicate for at least two incubation times, except for assays of hepatoma cells, when on occasions there was insufficient material. In such cases, single assays were performed at two incubation times.

Pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK)

Extraction of enzymes

Tissues were homogenised in 10 mM Tris-HCl, pH 7.4 containing

1 mM DTT, using an Ultra-Turrax homogeniser. The homogenates were centrifuged at 36,000 g, 4°, for 30 minutes using an MSE 18 High Speed refrigerated centrifuge. The supernatants were used for enzyme assays.

Cell extracts were prepared in 50 mM Tris-HCl, pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and 5 mM mercaptoethanol, by freezing and thawing three times in an acetone-dry ice bath. The extracts were centrifuged at 36,000 g, 4°, for 1 hour using an MSE 18 High Speed refrigerated centrifuge. The supernatants were used for enzyme assays.

Assays

The extracts contained particulate as well as cytosol enzymes and the total PC and PEPCK activities were measured in the tissues and cells. The assays for PC and PEPCK were similar in principle and measured the incorporation of ^{14}C-bicarbonate into oxaloacetate or malate, respectively, as shown below.

$$\text{PC:}\quad \text{pyruvate} + \text{ATP} + H^{14}CO_3^- \xrightleftharpoons[\text{AcCoA}]{Me^{2+}Me^{+}} {}^{14}\text{C-oxaloacetate} + \text{ADP} + P_i$$

$$\text{PEPCK:}\quad \text{phosphoenolpyruvate} + \text{IDP} + H^{14}CO_3^- \xrightleftharpoons{Me^{2+}} {}^{14}\text{C-oxaloacetate} + \text{ITP}$$

$$^{14}\text{C-oxaloacetate} + \text{NADH} + H^+ \xrightarrow[\text{MDH}]{} {}^{14}\text{C-malate} + NAD^+$$

Incorporation rates were proportional to both time and enzyme concentration for the incubation times and amounts of enzyme used.

All solutions were degassed before use and stored over soda lime to minimise the content of dissolved carbon dioxide.

Pyruvate carboxylase (PC)

The incubation medium was based on those of Henning and Seubert (1964) and Wimhurst and Manchester (1970). However, acetyl coenzyme A was not prepared *in situ* but included in the assay mixture, and ^{14}C-incorporation into oxaloacetate or malate was measured instead of ^{14}C-incorporation into citrate.

The substrate and cofactor concentrations used were shown by Wimhurst and Manchester (1970) to be optimal for rat liver pyruvate carboxylase.

The incubation medium contained 125 mM Tris-HCl, pH 7.4, 6.66 mM potassium pyruvate, 8.3 mM magnesium sulphate, 2.2 mM ATP, 0.75 mM acetyl coenzyme A and 50 mM sodium bicarbonate-^{14}C (0.013 µC/µmole). The reaction was initiated by addition of 0.1 ml of suitably diluted tissue homogenate or cell extract. The total volume of the reaction mixture was 0.3 ml and the final pH was 7.2 at 30°.

Incubation times (30°) ranged from 5 - 30 minutes depending on enzyme activity. Reactions were stopped by addition of 0.5 ml cold 10% trichloroacetic acid. Precipitated protein was removed after centrifugation at 50 g, at room temperature, for 5 minutes and nitrogen was bubbled through the supernate for 10 minutes to remove $^{14}CO_2$. An aliquot (0.4 ml) of the supernate was added to 10 ml Instagel in a scintillation vial and counted in a Packard Model 3390 liquid scintillation spectrometer using the Packard preset carbon-14 channel.

In the absence of acetyl coenzyme A or potassium pyruvate or if zero time controls were carried out (enzyme was added after the trichloroacetic acid) a blank incorporation of approximately 50 - 200 c.p.m. was obtained and this value was subtracted from all assay measurements. In most assays 200 - 2,000 c.p.m. above the blank values were incorporated into products.

Total counts were determined by adding 0.6 ml water to 0.2 ml reaction mixture and counting 0.2 or 0.4 ml aliquots of this mixture in 10 ml Instagel. The channels ratio method showed that samples were not quenched with respect to the totals.

Phosphoenolpyruvate carboxykinase (PEPCK)

The incubation medium was as described by Ballard and Hanson (1967) and was also similar to that of Chang and Lane (1966). It contained 100 mM imidazole-HCl, pH 6.6, 2 mM manganese chloride, 1 mM glutathione (reduced form), 1.25 mM IDP, 50 mM (0.04μC/μmole) sodium bicarbonate-^{14}C, 2.5 mM NADH, 3 mM phosphoenolpyruvate and 2 U malate dehydrogenase. The reaction was initiated by addition of 0.1 ml of suitably diluted tissue homogenate or cell extract. The total volume of the reaction mixture was 1 ml and the final pH was 6.9 - 7.1 at 30°.

Incubation times (30°) ranged from 2 to 10 minutes depending on enzyme activity. Reactions were stopped by addition of 0.5 ml cold 10% trichloroacetic acid and precipitated protein was removed by centrifugation at 50 g, at room temperature, for 5 minutes. Nitrogen was bubbled through the supernatant to remove any remaining $^{14}CO_2$. Aliquots (1 ml) were transferred to scintillation vials containing 10 ml Instagel and counted in a Packard Model 3390 liquid scintillation spectrometer using the Packard preset carbon-14 channel.

In the absence of IDP or PEP, or if zero time controls were carried out (enzyme was added after the trichloroacetic acid), a blank incorporation of about 300 - 500 c.p.m. was obtained and this value was subtracted from the assay value. In most assays 500 - 4,000 c.p.m. above the blank values were incorporated into products.

Total counts were determined by adding 0.6 ml water to 0.9 ml reaction mixture and counting 0.5 ml or 1 ml aliquots in 10 ml Instagel. The channels ratio method showed that samples were not quenched with respect to the totals.

Glucose-6-phosphatase (G6Pase)

Extraction of enzyme

Tissues were homogenised in ice-cold 0.25 M sucrose using a 5 - 6 strokes of a Potter-Elvehjem homogeniser (Nordlie and Snoke, 1967). Cell extracts were prepared as described on p. 83.

Assay

Glucose-6-phosphatase phosphohydrolase activity was determined by measuring inorganic phosphate released from glucose-6-phosphate.

$$\text{glucose-6-phosphate} + H_2O \xrightarrow{\text{G6Pase}} \text{glucose} + P_i$$

The assay mixture was as described by Nordlie and Arion (1966) and Nordlie and Snoke (1967) and contained 40 mM sodium cacodylate buffer, pH 6.5 and 20 mM glucose-6-phosphate. The reaction was initiated by addition of 0.1 ml of suitably diluted tissue homogenate or cell extract. The total volume of the reaction mixture was 1.5 ml. These conditions have been shown to be optimal for the rat liver enzyme (Nordlie, 1971).

Incubation times (30°) ranged from 5 - 60 minutes depending on enzyme activity. Reactions were stopped by addition of 1 ml ice-cold 10% trichloroacetic acid. The precipitated protein was removed by centrifugation at 50 g, at room temperature, for 5 minutes and inorganic phosphate present in the supernatant was determined by the method of Berenblum and Chain, as modified by Martin and Doty (Lindberg and Ernster, 1956).

Phosphate determination

Anhydrous disodium hydrogen orthophosphate was used to prepare a series of standards. Standards and samples of volume 3 ml, were mixed with 0.5 ml 5 M sulphuric acid, 5 ml isobutanol-benzene (1:1) and 0.5 ml 10% ammonium molybdate. The phases were allowed to separate and 1 ml or 2 ml aliquots (depending on the activity) of the organic layer were transferred to clean tubes and made up to 5 ml with 3.2% sulphuric acid in absolute ethanol. 0.5 ml of a 10% solution of stannous chloride in concentrated hydrochloric acid, which had been diluted two hundred times with 0.5 M sulphuric acid just before use, was added and the solutions mixed immediately. Optical density was measured at 640 nm using a Beckman DB spectrophotometer.

The phosphate standard curve was linear in the range used and the colour was stable for at least 4 hours. Reaction rates were linear with respect to time and amount of enzyme at the concentrations used.

Zero time (blank) values (enzyme was added after the trichloroacetic acid) were subtracted from values obtained for the samples.

Glycogen synthetase (GS)

Extraction of enzyme

Tissues were homogenised in 10 mM Tris-HCl, pH 7.4 containing 125 mM sodium fluoride (to inhibit conversion of GS a to GS b) and 5 mM DTT using 5 - 6 strokes of a Potter-Elvehjem homogeniser. After centrifugation at 5,000 g, 4°, for 10 minutes using an MSE 18 High Speed refrigerated centrifuge, the supernatant was used for assay.

Cell extracts were prepared in the same buffer by freezing and thawing three times in an acetone-dry bath and, after centrifugation as

above, the clear supernatant was used for assay.

Assay

Glycogen synthetase activity was assayed by measuring the incorporation of glucose into glycogen using uridine diphosphoglucose labelled with tritium.

$$(\text{glucose})_n + \text{UDPG-}^3\text{H} \longrightarrow (\text{glucose-}^3\text{H})_{(n+1)} + \text{UDP}$$

Glycogen synthetase exists in two enzymatically interconvertible forms, a phosphorylated form, GS b, which requires glucose-6-phosphate for activity and a dephosphorylated form, GS a, which is active in the absence of glucose-6-phosphate. Assays were carried out in the presence and absence of glucose-6-phosphate and the total activity (a + b) (with glucose-6-phosphate) minus synthetase a activity (without glucose-6-phosphate) was taken as the synthetase b activity.

The assay mixture was essentially that of Saheki *et al.* (1971) and Sato *et al.* (1973a), except that UDPG labelled with tritium instead of with carbon-14 was used. Substrate and cofactor concentrations used were optimal for rat liver GS (Sato *et al.* 1973a). The incubation medium contained 50 mM glycylglycine, pH 8.2, containing 30 mg rabbit liver glycogen/ml, 5 mM uridine diphosphoglucose-^{3}H, (0.03μC/μmole), 10 mM glucose-6-phosphate (for total (a + b) activity) and 100 mM sodium fluoride. The reaction was initiated by addition of 50 μl of suitably diluted tissue homogenate or cell extract. The total volume of the reaction mixture was 0.3 ml.

Incubation times (30°) ranged from 5 - 60 minutes depending on enzyme activity. Reactions were stopped by addition of 1 ml 30% potassium hydroxide containing 2 mg/ml rabbit liver glycogen and the assay tubes were heated for 5 minutes at 100° and then allowed to cool.

Glycogen was precipitated by the method of Steiner *et al.* (1965). One ml 95% ethanol was added to the mixture, which was then incubated for 1 hour in an ice bath. After centrifugation at 50 g, at room temperature, for 5 minutes, the supernatant was discarded and the precipitate dissolved in 0.5 ml 0.05 N HCl. One ml 95% ethanol was added and the mixture was again incubated for 1 hour in an ice bath, followed by centrifugation at 50 g, at room temperature, for 5 minutes. The supernatant was discarded and the precipitate dried at 60 - 65° and dissolved in 1.2 ml water. An aliquot (1 ml) was transferred to a scintillation vial containing 10 ml Instagel and counted in a Packard Model 3390 liquid scintillation spectrometer using the preset tritium channel.

Zero time controls were carried out (enzyme was added after the potassium hydroxide) and blank incorporations of the order 150 - 250 c.p.m. were obtained. The zero time values were subtracted from the assay values. In most assays 1,000 - 6,000 c.p.m. above the zero time values were incorporated into glycogen. Incorporation rates were proportional to both time and enzyme concentration for the incubation times and amounts of enzyme used.

Total counts were determined by adding 50 µl of the reaction mixture to 10 ml of Instagel and 1 ml of water in a scintillation vial. The channels ratio method showed that samples were not quenched with respect to the totals.

Enzyme activity

A unit of enzyme activity is defined as the amount of enzyme catalysing the conversion of 1 µmole of substrate to product per minute at 30° under the conditions specified above. Enzyme activity is expressed

as U/g wet weight of tissue or as mU/mg protein in tissues and as mU/mg protein in cell cultures.

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951). Suitable dilutions of samples were prepared in water and bovine plasma albumin was used to prepare a series of standards ranging from 0 to 150 µg/ml. Just before use, 1% copper sulphate, 2.7% sodium-potassium tartrate and a solution of 2% sodium carbonate in 0.1 N sodium hydroxide were mixed in the proportion 1:1:48. Five ml of this solution was added to 1 ml of sample or standard and after 10 - 20 minutes, 0.5 ml Folin-Ciocalteu reagent (diluted 1:2 in water) was added. After a further 30 minutes optical densities were determined at 750 nm using a Beckman DB spectrophotometer.

Statistical analysis of results

The levels obtained for livers, hepatomas and hepatoma cells were subjected to statistical analysis and expressed as mean values ± standard error of the mean. The significance of differences was determined by applying Student's t test ($p < 0.01$ was considered statistically significant).

Subcellular fractionation of human foetal livers

Subcellular fractionation of human foetal livers, using 5 g of liver, was carried out as described on p.173 and PEPCK activity was determined in the nuclear, mitochondrial and cytosol fractions. Glutamate dehydrogenase and lactate dehydrogenase were used as marker enzymes for the mitochondrial and cytosol fractions, respectively, and were assayed as described on p.170.

RESULTS

Stability of enzymes

Enzyme activities in fresh livers were compared with the activities in the same livers, which had either been kept at room temperature or which had been frozen at -20°. The results are shown in Table I. Pyruvate carboxylase (PC) was unstable both in livers maintained at room temperature and in frozen livers. Phosphoenolpyruvate carboxykinase was, however, much more stable to freezing. Glucose-6-phosphatase (G6Pase) was unstable in tissues maintained at room temperature, but was extremely stable to freezing. Glycogen synthetase (GS) was unstable in tissue maintained at room temperature, but in livers which had been frozen for two days, the activities were only slightly lower than the initial activities in the fresh livers. However, when tissues were frozen for longer periods, GS was much less stable and the proportion of GS a was slightly lower than in fresh tissue. In view of these results, PC, PEPCK and GS assays were carried out as soon as possible using fresh tissue. G6Pase activities were occasionally assayed at a later stage, using frozen material.

Since it was possible that PC was cold labile, the activities of PC and also of PEPCK in rat liver homogenates, which had been prepared and centrifuged at room temperature, were compared with the activities in homogenates of the same livers, which had been prepared and centrifuged at 4°. The homogenates maintained at room temperature had 81 and 90% of the PC and PEPCK activities, respectively, in homogenates maintained at 4°. Extracts used for determination of PC and PEPCK activities were therefore prepared at 4°.

On two occasions, washed rat hepatoma cells were frozen for 2 - 3 hours before extracts were prepared. Enzyme activities in extracts

TABLE I

STABILITY OF ENZYMES IN RAT AND HUMAN LIVERS

PYRUVATE CARBOXYLASE

TISSUE	CONDITIONS UNDER WHICH TISSUE WAS MAINTAINED		% INITIAL ACTIVITY IN FRESH TISSUE
Rat liver	Room temperature	3 hours	88
	Frozen	3 hours	82
Human foetal liver	Frozen	2 weeks	2

PHOSPHOENOLPYRUVATE CARBOXYKINASE

TISSUE	CONDITIONS UNDER WHICH TISSUE WAS MAINTAINED		% INITIAL ACTIVITY IN FRESH TISSUE
Rat liver	Room temperature	3 hours	94
	Frozen	1 month	90
Human foetal liver	Frozen	2 weeks	76

GLUCOSE-6-PHOSPHATASE

TISSUE	CONDITIONS UNDER WHICH TISSUE WAS MAINTAINED		% INITIAL ACTIVITY IN FRESH TISSUE
Rat liver	Room temperature	2 hours	65
	Frozen	3 weeks	97
Human adult liver	Frozen	3 weeks	95
Human foetal liver	Frozen	3 weeks	99

GLYCOGEN SYNTHETASE

TISSUE	CONDITIONS UNDER WHICH TISSUE WAS MAINTAINED		% INITIAL ACTIVITY IN FRESH TISSUE		
			(a + b)	a	b
Rat liver	Room temperature	2 hours	62	65	59
	Frozen	2 days	99	101	97
	Frozen	6 days	52	39	64
Human adult liver	Frozen	2 days	95	96	95
	Frozen	2 months	28	26	28
Human foetal liver	Frozen	2 weeks	26	12	73

prepared from the frozen cells were compared with the activities in extracts prepared from cells which had been grown under the same conditions, at the same time, but which had not been frozen. In the frozen cells, PC activities were 65% and 47% and PEPCK activities 86% and 76% of the activities in the cells which were not frozen. G6Pase and total GS activities were slightly higher in the frozen cells; G6Pase levels were higher by 5% and 16% and total GS levels by 13% and 18%. GS a activity was, however, lower in the frozen cells, considerably so in one case, and was 14.6% and 77% of the activity in cells which were not frozen.

Enzyme levels

The activities of the enzymes, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and glycogen synthetase (GS) were determined in livers, hepatomas and hepatoma cells and the results (expressed as mU/mg protein) are shown in Figs. 1 and 2. A line is used to join the activity of the enzyme in hepatoma tissue (H) to that of its corresponding host tissue (HL). Total GS activity (a + b) was determined in the presence of glucose-6-phosphate and GS a activity in the absence of glucose-6-phosphate. GS b activity was taken as the difference between the total GS activity and GS a activity. The activity ratios $\frac{a}{(a + b)}$ were also calculated (Fig. 2). Mean values (± S.E.M.) expressed as mU/mg protein or as U/gm wet weight of tissue are listed in Tables II - V. Student's t test was used to determine the significance of differences between means and the p values obtained are included in the Tables.

Enzyme levels in rat livers, hepatomas and hepatoma cell cultures

It can be seen from Figs. 1 and 2 and Tables II and III that

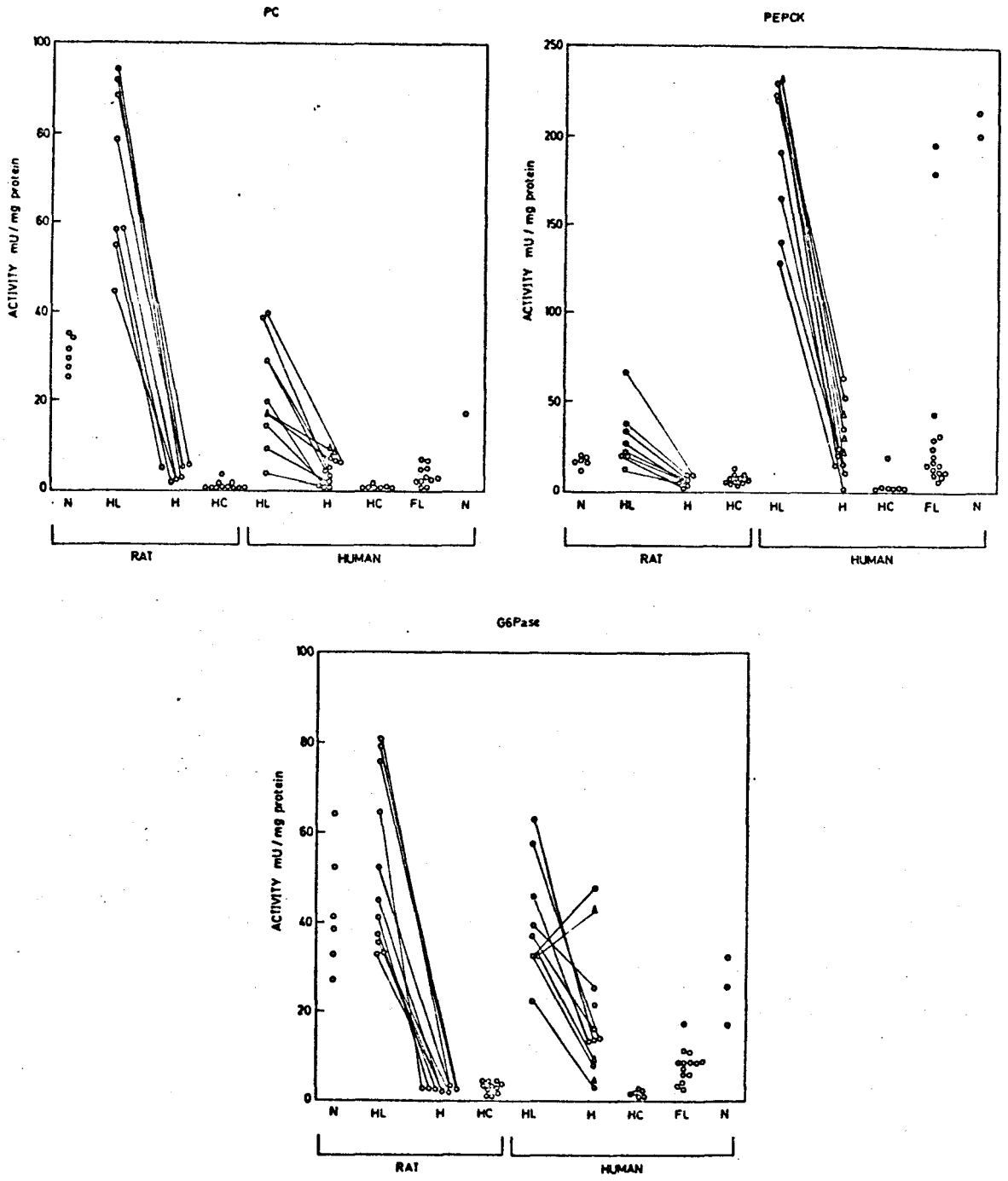


FIGURE 1. Activities of the gluconeogenic enzymes, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in rat and human livers (N - normal liver, HL - host liver, FL - foetal liver), hepatomas (H) and hepatoma cell cultures (HC). A line joins the levels in host livers to those of the corresponding hepatomas. Δ - Surgical tissue.

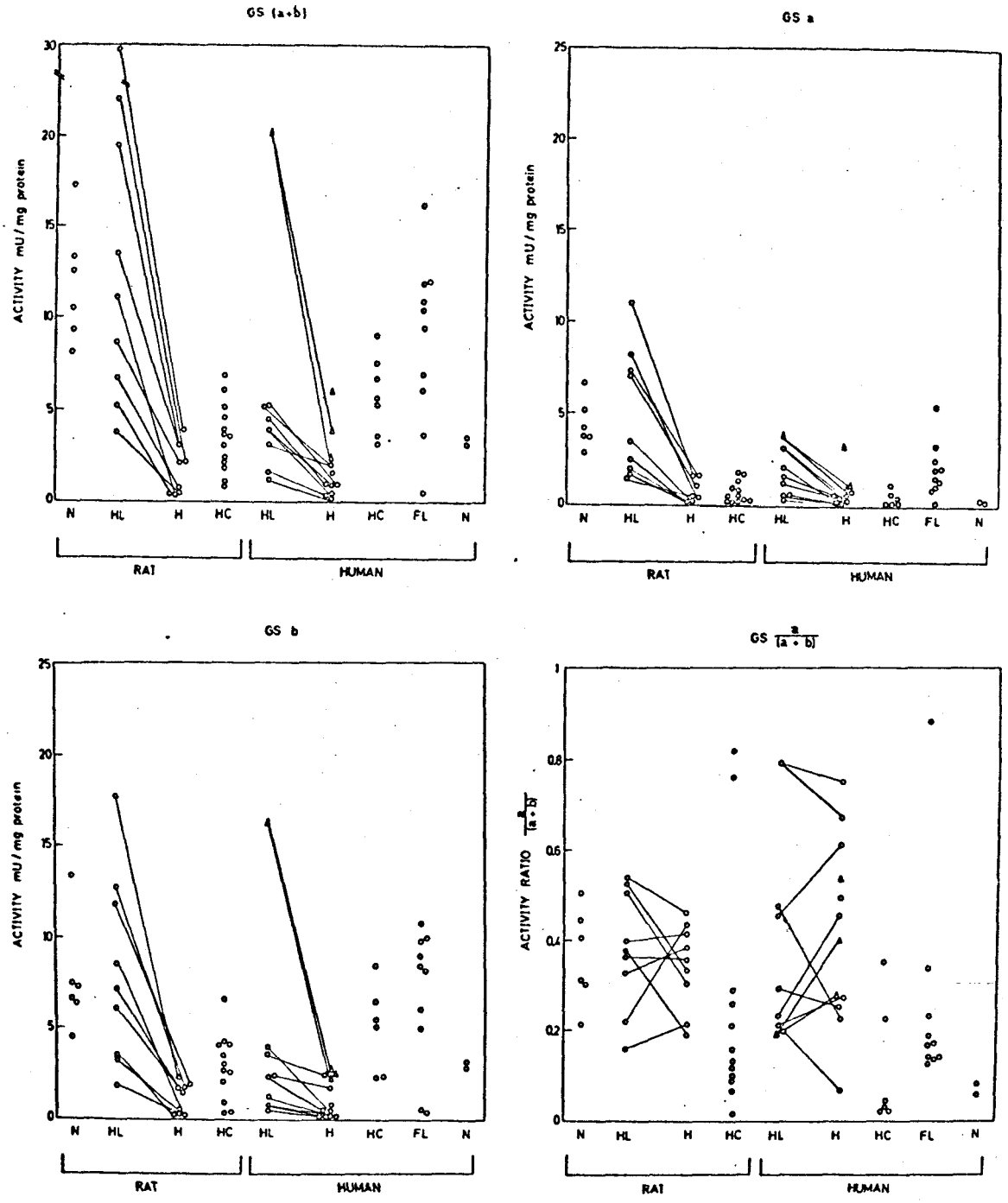


FIGURE 2. Glycogen synthetase (GS) activities in rat and human livers (N - normal liver, HL - host liver, FL - foetal liver), hepatomas (H) and hepatoma cell cultures (HC). A line joins the levels in host livers to those of corresponding hepatomas. The difference between total GS activity (a + b), measured in the presence of glucose-6-phosphate, and GS a activity, measured in the absence of glucose-6-phosphate, was taken as GS b activity and the activity ratio $\frac{a}{a+b}$ is also shown. Δ - Surgical tissue.

TABLE II

ACTIVITIES OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE IN RAT LIVERS, HEPATOMAS AND HEPATOMA CELL CULTURES

ACTIVITIES EXPRESSED AS mU/mg PROTEIN (MEAN ± S.E.M.)

ENZYME	NORMAL LIVER (6)*	HOST LIVER	HEPATOMA	HEPATOMA % ACTIVITY OF HOST (HOST = 100%)	HEPATOMA CELLS	p VALUES ON STATISTICAL ANALYSIS			
						HOST vs. NORMAL	HOST vs. HEPATOMA	NORMAL vs. HEPATOMA	HEPATOMA vs. HEPATOMA CELLS
Pyruvate carboxylase	30.6 ± 1.5	77.1 ± 8.6 (9)	3.3 ± 0.5 (9)	4.49 ± 0.73 (9)	1.04 ± 0.29 (11)	<0.0005	<0.0005	<0.0005	0.0005<p<0.0025
Phosphoenolpyruvate carboxykinase	16.7 ± 1.3	29.3 ± 6.0 (9)	6.3 ± 0.7 (9)	24.9 ± 3.2 (9)	6.91 ± 0.78 (11)	NS+	0.0005<p<0.0025	<0.0005	NS
Glucose-6-phosphatase	42.5 ± 5.5	52.5 ± 4.5 (14)	2.1 ± 0.1 (14)	3.37 ± 0.63 (14)	2.84 ± 0.36 (12)	NS	<0.0005	<0.0005	NS
Glycogen synthetase (a + b)	11.8 ± 1.3	13.4 ± 2.9 (9)	1.82 ± 0.45 (9)	13.5 ± 2.3 (9)	3.49 ± 0.51 (13)	NS	0.0005<p<0.0025	<0.0005	NS
a	4.17 ± 0.58	5.1 ± 1.2 (9)	0.63 ± 0.19 (9)	12.9 ± 2.9 (9)	0.74 ± 0.17 (12)	NS	0.0005<p<0.0025	<0.0005	NS
b	7.66 ± 1.23	8.3 ± 1.7 (9)	1.19 ± 0.28 (9)	15.1 ± 2.5 (9)	2.97 ± 0.54 (12)	NS	0.0005<p<0.0025	<0.0005	0.0025<p<0.005
$\frac{a}{(a + b)}$	0.36 ± 0.04	0.38 ± 0.04 (9)	0.34 ± 0.03 (9)		0.25 ± 0.07 (12)	NS	NS	NS	NS

*Numbers in parentheses are numbers of determinations.

+NS - not significant, p >0.01.

TABLE III

ACTIVITIES OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE IN RAT LIVERS AND HEPATOMAS

ACTIVITIES EXPRESSED AS U/gm WET WEIGHT OF TISSUE (MEAN ± S.E.M.)

ENZYME	NORMAL LIVER (6)*	HOST LIVER	HEPATOMA	HEPATOMA % ACTIVITY OF HOST (HOST = 100%)	p VALUES ON STATISTICAL ANALYSIS		
					HOST vs. NORMAL	HOST vs. HEPATOMA	NORMAL vs. HEPATOMA
Pyruvate carboxylase	3.23 ± 0.11	7.7 ± 0.7 (9)	0.19 ± 0.03 (9)	2.71 ± 0.49 (9)	<0.0005	<0.0005	<0.0005
Phosphoenolpyruvate carboxykinase	1.79 ± 0.13	3.26 ± 0.61 (9)	0.33 ± 0.03 (9)	12.46 ± 1.22 (9)	NS+	<0.0005	<0.0005
Glucose-6-phosphatase	7.72 ± 0.59	9.51 ± 0.69 (14)	0.21 ± 0.02 (14)	2.28 ± 0.2 (14)	NS	<0.0005	<0.0005
Glycogen synthetase (a + b)	0.85 ± 0.11	0.8 ± 0.1 (9)	0.064 ± 0.015 (9)	7.74 ± 1.16 (9)	NS	<0.0005	<0.0005
a	0.29 ± 0.03	0.31 ± 0.07 (9)	0.025 ± 0.007 (9)	8.11 ± 1.81 (9)	NS	<0.0005	<0.0005
b	0.56 ± 0.09	0.49 ± 0.09 (9)	0.039 ± 0.008 (9)	8.14 ± 1.13 (9)	NS	<0.0005	<0.0005
$\frac{a}{(a + b)}$	0.36 ± 0.04	0.38 ± 0.04 (9)	0.34 ± 0.03 (9)		NS	NS	NS

*Numbers in parentheses are numbers of determinations.

+NS - not significant, $p > 0.01$.

TABLE IV

ACTIVITIES OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE IN HUMAN LIVERS, HEPATOMAS AND HEPATOMA CELL CULTURES

ACTIVITIES EXPRESSED AS mU/mg PROTEIN (MEAN ± S.E.M.)

	AUTOPSY TISSUE						SURGICAL TISSUE			
ENZYME	FOETAL LIVER	NORMAL LIVER	HOST LIVER (7)	HEPATOMA (9)	HEPATOMA (7)· % ACTIVITY OF HOST (HOST = 100%)	p VALUES HOST vs. HEPATOMA	HOST LIVER (1)	HEPATOMA (3)	HEPATOMA CELLS	p VALUES HEPATOMA (AUTOPSY) vs. HEPATOMA CELLS
Pyruvate carboxylase	2.96 ± 0.71 (11)*	17.7 (1)	21.9 ± 5.4	3.02 ± 0.82	12.3 ± 3.0	0.0005<p<0.0025	16.7	8.5 ± 0.7	0.73 ± 0.13 (7)	0.005<p<0.01
Phosphoenolpyruvate carboxykinase	39.3 ± 14.6 (16)	206.1 ± 7.1 (2)	186.8 ± 11.9	26.4 ± 6.8	13.2 ± 3.1	<0.0005	232.4	31.9 ± 5.8	4.98 ± 2.27 (7)	0.0005<p<0.0025
Glucose-6-phosphatase	8.33 ± 1.06 (14)	24.9 ± 4.42 (3)	42.5 ± 5.4	18.0 ± 4.3	45.8 ± 15.6	0.0005<p<0.0025	32.4	18.9 ± 12.1	1.74 ± 0.32 (6)	0.0005<p<0.0025
Glycogen synthetase (a + b)	8.87 ± 1.43 (10)	3.27 ± 0.15 (2)	3.55 ± 0.62	1.05 ± 0.24	26.7 ± 6.9	0.0005<p<0.0025	20.3	4.05 ± 1.0	5.87 ± 0.79 (7)	<0.0005
a	2.04 ± 0.48 (10)	0.23 ± 0.03 (2)	1.36 ± 0.38	0.36 ± 0.08	22.4 ± 4.1	0.005<p<0.01	3.8	1.75 ± 0.7	0.46 ± 0.18 (6)	NS+
b	5.84 ± 1.19 (10)	3.04 ± 0.17 (2)	2.19 ± 0.51	0.69 ± 0.22	30.2 ± 8.3	0.005<p<0.01	16.5	2.58 ± 0.17	5.14 ± 0.99 (6)	<0.0005
$\frac{a}{(a + b)}$	0.25 ± 0.07 (10)	0.07 ± 0.01 (2)	0.38 ± 0.08	0.42 ± 0.07		NS	0.19	0.40 ± 0.07	0.11 ± 0.06 (6)	0.0025<p<0.005

*Numbers in parentheses are numbers of determinations.

+NS - not significant, p > 0.01.

TABLE V

ACTIVITIES OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE IN HUMAN LIVERS AND HEPATOMAS

ACTIVITIES EXPRESSED AS U/gm WET WEIGHT OF LIVER (MEAN ± S.E.M.)

ENZYME	AUTOPSY TISSUE						SURGICAL TISSUE	
	FOETAL LIVER	NORMAL LIVER	HOST LIVER (7)	HEPATOMA (9)	HEPATOMA (7) % ACTIVITY OF HOST (HOST = 100%)	p VALUES HOST vs. HEPATOMA	HOST LIVER (1)	HEPATOMA (5)
Pyruvate carboxylase	0.21 ± 0.04 (11)[x]	1.27 (1)	1.79 ± 0.41	0.15 ± 0.05	7.53 ± 2.17	0.0005<p<0.0025	1.89	0.56 ± 0.08
Phosphoenolpyruvate carboxykinase	2.59 ± 1.06 (16)	17.9 ± 2.6 (2)	15.7 ± 1.3	1.38 ± 0.43	8.36 ± 2.58	<0.0005	26.3	2.13 ± 0.56
Glucose-6-phosphatase	0.94 ± 0.13 (14)	4.23 ± 0.55 (3)	5.84 ± 0.51	1.69 ± 0.43	30.4 ± 10.8	<0.0005	5.13	2.40 ± 1.56
Glycogen synthetase (a + b)	0.33 ± 0.07 (10)	0.28 ± 0.003 (2)	0.32 ± 0.07	0.05 ± 0.01	14.8 ± 4.2	.0005<p<0.0025	1.98	0.27 ± 0.09
a	0.08 ± 0.02 (10)	0.02 ± 0.003 (2)	0.12 ± 0.03	0.02 ± 0.003	2.04 ± 2.31	0.0005<p<0.0025	0.37	0.12 ± 0.06
b	0.25 ± 0.06 (10)	0.26 ± 0.006 (2)	0.20 ± 0.06	0.03 ± 0.01	16.7 ± 4.9	0.005<p<0.01	1.61	0.15 ± 0.04
$\frac{a}{(a + b)}$	0.28 ± 0.11 (10)	0.07 ± 0.01 (2)	0.38 ± 0.08	0.42 ± 0.07		NS[+]	0.19	0.41 ± 0.07

[x] Numbers in parentheses are numbers of determinations.

[+] NS - not significant, $p > 0.01$,

in normal rat livers, the levels of PC were significantly lower than in host livers, whereas the levels of PEPCK, G6Pase and GS did not differ significantly from those in host livers. In hepatomas the levels of all four enzymes were always lower than those of the corresponding host tissue as shown in Figs. 1 and 2, and the mean hepatoma values were significantly lower than those of normal or host liver as shown in Tables II and III. The values obtained when the levels of the enzymes in hepatomas were expressed as percentages of the levels in the corresponding host livers are also shown in Tables II and III. Activities of both a and b forms of GS were lower in hepatomas than in host livers, though the proportions of the two forms were not significantly changed.

The levels of PC, PEPCK, G6Pase and GS in the two rat hepatoma cell lines, (the one supplied by Professor O.W. Prozesky and the other supplied by Dr. L.D. Nourse) grown under the conditions described on p. 82, fell into the same range and are included together in Figs. 1 and 2 and Table II. Student's t test showed that in the hepatoma cells, the levels of PC were significantly lower than in hepatoma tissue, whereas the levels of PEPCK and G6Pase were not significantly different from hepatoma levels. Total GS activities in the hepatoma cells were higher than in hepatoma tissue (though this was not statistically significant). The activities of GS a were similar in hepatoma cells and hepatoma tissue, but the levels of GS b were significantly higher in the cells. In general, there was a greater proportion of GS b in the hepatoma cells, though in two cases the proportion of GS a was high (see Fig. 2). The mean ratios $\frac{a}{(a + b)}$ did not differ significantly from the values for normal liver, host liver or hepatoma.

Enzyme levels in human livers, hepatomas and hepatoma cell culture

As shown in Figs. 1 and 2 the levels of PC were lower in human

livers than in rat livers whereas PEPCK levels were higher. G6Pase levels in human livers were similar to those in rat livers and, in general, GS levels were lower.

The levels of PC, PEPCK, G6Pase and GS in normal livers obtained at autopsy were within the ranges of the levels in host livers also obtained at autopsy. There are, however, only a few values for normal livers owing to difficulties in obtaining this material.

In hepatomas obtained at autopsy, the levels of PC, PEPCK and GS were, in all cases, lower than in the corresponding host livers, as shown in Figs. 1 and 2. G6Pase levels were also lower in hepatomas than in the corresponding host tissues, except in one of two samples of the same hepatoma tissue, for which a higher value was obtained (see also Table VI). Mean values of the enzyme levels in host livers and hepatomas and the values obtained when hepatoma levels were expressed as percentages of the corresponding host liver levels are given in Tables IV and V. For all four enzymes, the mean hepatoma values were significantly lower than the mean host liver values. Activities of both a and b forms of GS were lower in hepatomas than in host livers but the relative amounts of GS a and GS b were not significantly changed.

On two occasions, tissue was obtained during surgery and the data are included in Figs. 1 and 2 and Tables IV and V. In the one case, where both hepatoma and the corresponding host liver were available for assay, the levels of PC, PEPCK and GS (a and b forms) were found to be lower in the hepatoma than in the host liver, as in autopsy tissue. For G6Pase, a higher level was obtained for one of two samples of tissue (see also Table VI). The levels of PC, PEPCK and G6Pase in the surgical host liver and hepatomas were comparable with those in autopsy livers and

hepatomas respectively. The levels of GS (a + b) and GS b were considerably higher (more than five times) in the host liver than in autopsy livers, whilst GS a activity was only slightly higher. The GS (a + b), GS a and GS b activities were somewhat higher in surgical hepatomas than in autoopsy hepatomas.

Table VI shows the results obtained when different regions of hepatoma were assayed. The levels of PC, PEPCK and GS (a and b forms) were lower in hepatoma adjacent to the host liver, and lower still in hepatoma further removed from the host liver, than in host liver, when either surgical or autopsy material was used. G6Pase levels were higher in hepatoma adjacent to the host liver than in host liver, but when hepatoma further removed from the host liver was assayed the levels were lower.

The levels of the four enzymes in the human hepatoma cell line are shown in Figs. 1 and 2 and mean values are given in Table IV. Comparison of the mean values for the cell line with mean autopsy hepatoma values, using Student's t test, showed that PC, PEPCK and G6Pase levels were significantly lower in the cells than in hepatomas, whereas GS (a + b) and GS b levels were significantly higher. There was no significant difference between GS a activity in the cells and in autopsy hepatomas although proportion of GS a was lower in the cells.

Enzyme levels in human foetal livers

All four enzymes had measurable activity in livers of human foetuses ranging from 13 to 28 weeks gestational age. The levels are shown in Figs. 1 and 2 and mean values in Tables IV and V. The levels of PC and G6Pase were lower in foetal livers than in adult livers and were often comparable with human hepatoma levels. PEPCK levels were also low,

TABLE VI

ACTIVITIES* OF KEY GLUCONEOGENIC ENZYMES AND GLYCOGEN SYNTHETASE

IN HUMAN HOST LIVER AND DIFFERENT REGIONS OF HEPATOMA.

	ENZYME	HOST LIVER	HEPATOMA TISSUE ADJACENT TO HOST LIVER	HEPATOMA TISSUE FURTHER REMOVED FROM HOST LIVER
SURGICAL TISSUE	Pyruvate carboxylase	16.7	9.27	7.08
	Phosphoenolpyruvate carboxykinase	232.4	42.6	22.5
	Glucose-6-phosphatase	32.4	43.0	9.32
	Glycogen synthetase (a+b)	20.3	3.98	2.34
	" " a	3.8	1.13	0.96
	" " b	16.5	2.85	1.38
	" " $\frac{a}{(a+b)}$	0.19	0.28	0.40
AUTOPSY TISSUE	Pyruvate carboxylase	28.6	4.48	4.02
	Phosphoenolpyruvate carboxykinase	230.2	63.9	24.2
	Glucose-6-phosphatase	32.4	47.7	7.82
	Glycogen synthetase (a+b)	3.98	0.83	0.49
	" " a	3.14	0.55	0.37
	" " b	0.84	0.28	0.12
	" " $\frac{a}{(a+b)}$	0.79	0.67	0.75

*Activities are expressed as mU/mg protein.

with the exception of two values obtained for spontaneously aborted twin foetuses. The levels of GS varied over a wide range and included values higher than those of adult human liver. There was a greater proportion of GS b except in one case where the activity ratio $\frac{a}{(a + b)}$ was 0.88.

Enzyme activities in foetal livers are plotted against the crown to rump length of the foetus in Fig. 3. Levels in livers of twin foetuses are shown as solid circles. A crown to rump length of 10 cm corresponds approximately to a foetus of 15 weeks gestation and a crown to rump length of 25 cm to a foetus of 28 weeks gestation. There did not seem to be any definite correlation of PC, PEPCK or G6Pase activity with foetal length or age, although PC levels seemed somewhat higher in 14 - 16 cm foetuses. PEPCK levels were considerably higher in livers of the twin foetuses. The activities of GS (a + b), GS a and GS b appeared to increase as foetal length increased from 9 cm to 20 cm (approximately 24 weeks) and GS b activity then decreased. The proportion of GS a was much higher in the liver of a 25 cm foetus than in livers of smaller foetuses.

Subcellular distribution of PEPCK in human foetal livers

Activities of PEPCK and the marker enzymes glutamate dehydrogenase and lactate dehydrogenase in nuclear, mitochondrial and cytosol fractions of human foetal livers of approximately 17 and 21 - 22 weeks gestational age are given in Table VII. Only a small proportion of glutamate dehydrogenase activity was present in the cytosol fractions indicating negligible leakage from the mitochondria. The mitochondria were hardly contaminated by cytosol enzymes as indicated by lactate dehydrogenase activities. The greatest proportion of PEPCK activity was present in the mitochondrial fractions (67.1 and 79.2%) with some activity in the cytosol (25.2 and 14.7%).

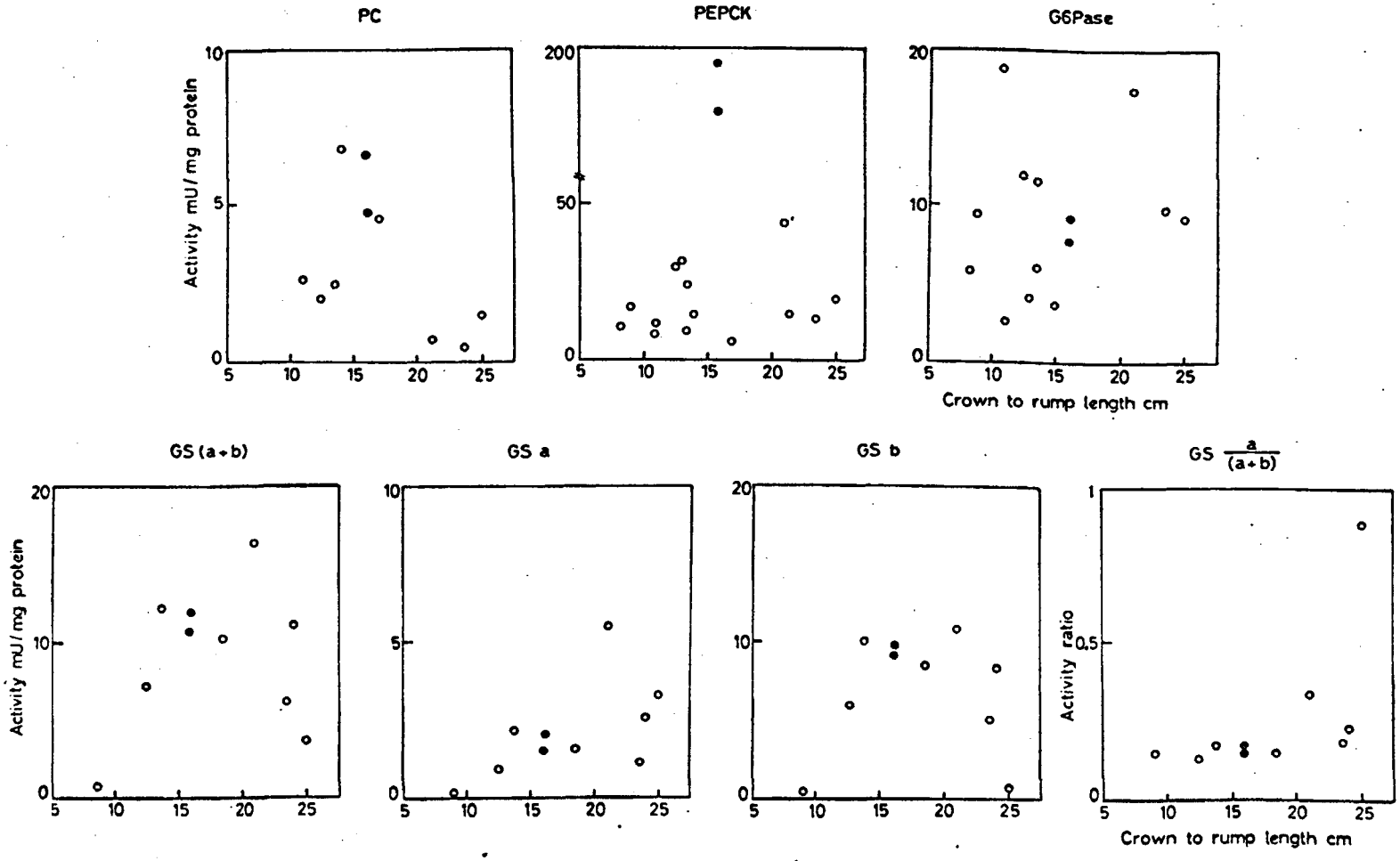


FIGURE 3. Activities of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and glycogen synthetase (GS) in human foetal livers as a function of crown to rump length of the foetus. Gestational age varied from approximately 13 weeks (8 cm) to 28 weeks (25 cm). o - Spontaneously aborted foetuses; • - therapeutically aborted twin foetuses.

TABLE VII

SUBCELLULAR DISTRIBUTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN HUMAN FOETAL LIVER

Subcellular fractions were prepared by differential centrifugation. Glutamate dehydrogenase and lactate dehydrogenase were used as marker enzymes for the mitochondrial and cytosol fractions, respectively.

APPROXIMATE AGE OF FOETUS	ENZYME	SPECIFIC ACTIVITY OF HOMOGENATE[+]	% RECOVERED ACTIVITY[x]			% RECOVERY[†]
			NUCLEI	MITOCHONDRIA	CYTOSOL	
17 weeks	Phosphoenolpyruvate carboxykinase	14.9	7.7	67.1	25.2	81.1
	Glutamate dehydrogenase	71.3	8.0	89.7	2.3	90.8
	Lactate dehydrogenase	1200.0	1.3	2.8	95.9	99.3
21-22 weeks	Phosphoenolpyruvate carboxykinase	5.8	6.1	79.2	14.7	91.8
	Glutamate dehydrogenase	51.0	8.3	88.1	3.6	93.6
	Lactate dehydrogenase	1360.0	1.4	2.3	96.3	94.4

[+] Specific activity expressed as mU/mg protein.

[x] Recovered activity = sum of activities in nuclear, mitochondrial and cytosol fractions. Phosphoenolpyruvate carboxykinase activity was not detected in microsomal fractions.

[†] % Recovery = $\frac{100 \times \text{sum of activities in nuclear, mitochondrial and cytosol fractions}}{\text{total activity in homogenate}}$

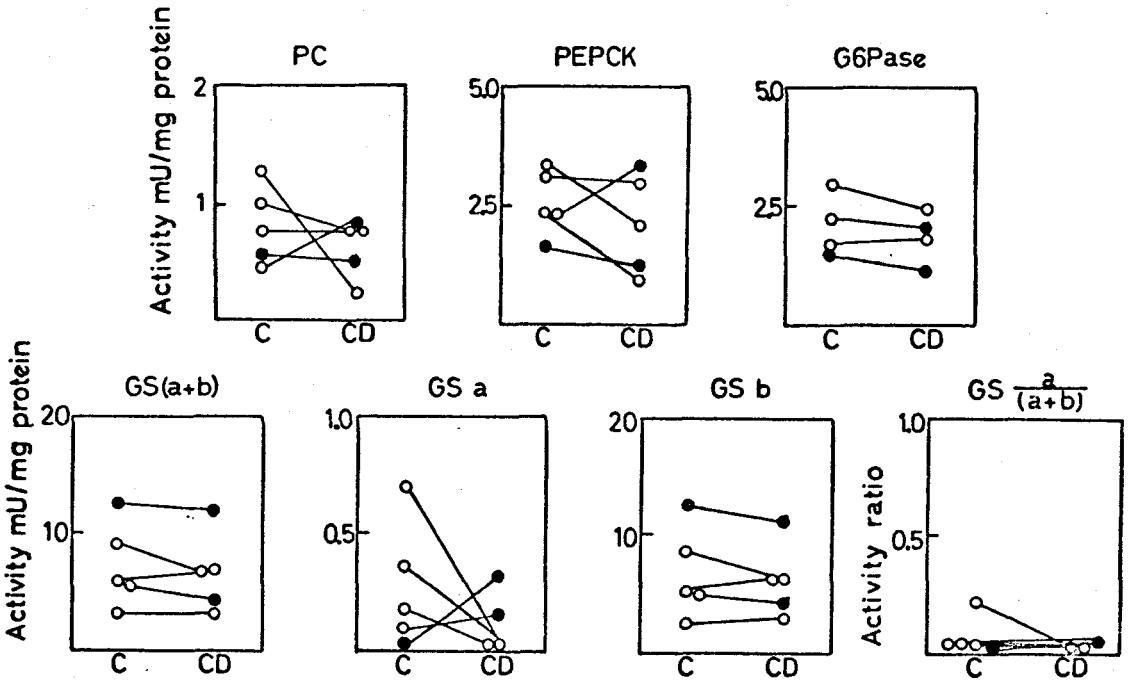


FIGURE 4. Activities of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and glycogen synthetase (GS) in human hepatoma cells grown in the absence of dexamethazone (C) and in the corresponding cells grown in the presence of 1 μM dexamethazone (CD). In some cases serum was omitted from the medium (●).

Effect of dexamethazone on enzyme levels in human hepatoma cells

Human hepatoma cells were split into two groups 18-24 hours before harvesting and to one group 1 μM dexamethazone (Decadron) was added. After harvesting, enzyme levels were determined in the two groups of cells and the results obtained on five different occasions are shown in Fig. 4. A line is used to join the activity of the enzyme in the control cells (C) to that in the corresponding cells grown in the presence of dexamethazone (CD). Foetal calf serum was omitted from the serum on three occasions as indicated by solid circles. It is apparent from Fig. 4 that there were no consistent changes in the enzyme levels in the cells grown in the presence of dexamethazone, whether or not serum was omitted from the medium.

DISCUSSION

The activities of the gluconeogenic enzymes, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in normal rat livers were comparable with those obtained by other workers. The mean value 3.23 ± 0.11 U/g liver for total pyruvate carboxylase activity was similar to the value 2.99 ± 0.22 U/g liver reported by Wimhurst and Manchester (1973), although higher values have been reported by Ballard and Hanson (1967) (12 U/g liver) and Chang and Morris (1973) (4.92 ± 0.21 U/g liver for mitochondrial PC). Total PEPCK activity, measured in the direction of oxaloacetate synthesis, in normal rat livers was 1.79 ± 0.13 U/g liver compared with values 2.38, 3.19 ± 0.26 and 1.47 ± 0.08 U/g liver obtained by Ballard and Hanson (1967), Wimhurst and Manchester (1973), and Pogson and Smith (1975), respectively. In normal rat livers, G6Pase activity was 7.72 ± 0.59 U/g liver (42.5 ± 5.5 mU/mg protein) compared with values of 5.1 ± 1.0 and 10.6 ± 0.7 U/g liver and 35.8 ± 2.2 mU/mg protein obtained by Sillero *et al.* (1969), Wimhurst and Manchester (1973) and Gutman *et al.* (1969), respectively.

The activity of PC was higher in rat host livers than in normal livers, in contrast to the findings of Chang and Morris (1973), who obtained levels lower than or similar to those of normal livers in a series of host livers corresponding to tumours of different growth rate. The activities of PEPCK and G6Pase in normal rat livers did not differ significantly from those in host livers. Weber and Cantero (1955a) found similar levels of G6Pase in normal livers and livers of rats bearing Novikoff hepatomas.

In rat hepatomas, the levels of PC, PEPCK and G6Pase were significantly lower than in normal or host livers indicating reduced

gluconeogenesis in the tumour tissue. Studies carried out by others on Morris hepatomas, Yoshida hepatomas and 3'-MeDAB-induced hepatomas have also shown the activities of these enzymes to be reduced in the tumours and a correlation has been made with growth rate, the faster the growth rate the lower the enzyme activity (Weber and Cantero, 1955a; Pitot, 1960; Weber and Morris, 1963; Sugimura *et al.*, 1966; Weber, 1963, 1966, 1974). However, in a recent study (Chang and Morris, 1973) it was shown that the levels of mitochondrial PC in a series of hepatomas did not correlate well with growth rate. The levels obtained for the 3'-MeDAB-induced hepatoma used in this study, when correlated with growth rate according to Weber (1966, 1974), are consistent with those of fairly fast-growing tumours. Reduced enzyme levels in hepatomas could result either from reduced synthesis of enzyme protein or from an alteration of the enzyme protein itself. Chang and Morris (1973) have shown that the enzymatic and immunological properties of PC in rat liver and hepatoma are very similar and therefore reduced activity in the tumour is probably a result of decreased protein synthesis. Whether this is also the case for PEPCK and G6Pase is not known.

The levels of glycogen synthetase (GS) in normal rat livers and host livers were similar. The activities of GS b in normal livers (0.56 ± 0.09 U/g liver) and host livers (0.49 ± 0.09 U/g liver) were lower than those obtained by Sato *et al.* (1973a) for normal and host livers (1.58 and 1.62 U/g liver, respectively) but the activities of GS a were comparable.

Activities of both a and b forms of GS were lower in rat hepatomas than in normal or host livers. The lower levels of GS a activity could indicate a reduction in the rate of glycogen synthesis in the tumours. The b form of GS was predominant in normal livers, host livers and hepatomas, which is consistent with the findings of Nigam (1967), Sato *et al.* (1973a)

and Lea *et al.* (1972), although a few hepatomas and muscle have been shown to have slightly greater proportion of GS a (Sato *et al.*, 1973a). Studies carried out by others have shown that levels of glycogen and GS decrease in the majority of experimental hepatomas, and that glycogen metabolism in fast-growing hepatomas differs to a greater extent from that in normal rat liver than it does in slow-growing hepatomas (Saheki *et al.*, 1971; Lea *et al.*, 1972; Misheneva *et al.*, 1973; Sato *et al.*, 1973a, 1973b). It has been shown that the altered GS activity in fast-growing, poorly-differentiated rat hepatomas is also associated with the replacement of the liver isozyme by a non-hepatic form (probably that of muscle) in the tumour (Sato *et al.*, 1972a, 1973a, 1973b).

In human livers, PC and GS levels were generally lower and PEPCK levels higher than in rat livers while G6Pase levels were similar. Species differences in the activities of hepatic PC and PEPCK have been reported by others. Wieland *et al.* (1968), Brech *et al.* (1970) and Söling *et al.* (1970) have also found levels of PC to be lower in human livers than rat livers but Brech *et al.* (1970) obtained similar values for PEPCK activity in livers of both species. The levels of PC in guinea pig livers have been shown to be similar to those in rat livers, whereas PEPCK levels were higher and the activity ratio $\frac{PC}{PEPCK}$ was found to be lower in both human and guinea pig livers than in rat livers (Söling *et al.* 1970).

Owing to difficulties in obtaining material, the data reported here represent only a few values for the enzyme activities in normal human livers, hence a satisfactory comparison of normal and host liver levels was not possible. However, the levels obtained for normal livers were within the ranges of the host liver values, which is consistent with the findings of Yeung *et al.* (1973) for PC, PEPCK and G6Pase.

The mean value for PC activity in human host livers obtained at autopsy was 1.79 ± 0.41 U/g liver as compared with the values 1.1 ± 0.7 and 3.0 ± 0.6 U/g liver reported by Wieland *et al.* (1968) and Räihä and Lindros (1969), respectively, for normal human livers obtained during surgery. The activity of PEPCK in the human host livers was 15.7 ± 1.3 U/g liver as compared with the value 9.1 ± 1.8 U/g liver obtained by Räihä and Lindros (1969) for normal surgical livers. The value 5.84 ± 0.51 U/g liver for G6Pase activity in human host livers was within the range of values (2 - 13 U/g liver) reported by others for normal human livers obtained during surgery (Schmidt and Schmidt, 1970). Total GS activity (0.32 ± 0.07 U/g liver) was lower than the range 1.4 - 3.1 U/g liver reported by Lewis *et al.* (1963).

Since the levels of PC, PEPCK and G6Pase in autopsy tissue were comparable with those in surgical tissue and similar to values obtained by others using surgical material, it would appear that there was little loss of activity of these enzymes during the time which elapsed between death and the assays being carried out. The activity of GS b was considerably lower in autopsy tissue than in surgical tissue and total GS values were lower than the values obtained by others using surgical material. This could be due to the instability of the b form of the enzyme or to the dietary state of the individuals from whom tissues were obtained.

The activities of PC, PEPCK, G6Pase and GS (both a and b forms) were significantly reduced in human hepatomas, when compared with the corresponding host liver levels, perhaps indicating reduced rates of gluconeogenesis in the human tumour, as in the fairly fast-growing 3'-MeDAB-induced rat hepatoma. In human hepatomas, the levels of PC, PEPCK and G6Pase were often comparable with those in foetal livers; the levels of GS (both forms) were, in general, lower than in foetal livers. The reduced levels of PC, PEPCK and G6Pase in human hepatomas are consistent

with the results of work (published while these studies were underway) carried out by Yeung *et al.* (1973) in Hong-Kong. The properties of PC, PEPCK, G6Pase and GS in human tissues have not been studied and it is therefore not known whether the lower activities in hepatomas result from the presence of non-hepatic forms of the enzymes or merely from reduced enzyme protein synthesis.

The activities of PC, PEPCK and G6Pase in rat and human hepatoma cells were comparable to or lower than the levels in the respective tumours, suggesting a low rate of gluconeogenesis as might be expected in rapidly-dividing cells derived from fairly fast-growing hepatomas.

Similarly, levels of G6Pase, in cell lines or suspensions, established from spontaneous mouse mammary carcinomas and uterine sarcomas and from thioacetamide-induced mouse hepatomas, have been shown to be similar to or lower than the levels in the corresponding tumour tissues (Bhide, 1970; Wilson, 1973). Levels of PEPCK, however, in Reuber H35 hepatoma cells (derived from a minimal deviation hepatoma) and $M_1H_1C_1$ hepatoma cells were shown to be similar to those of normal rat livers, whereas in HTC hepatoma cells (derived from Morris hepatoma 7288-C) and RLC rat liver cells, the levels were lower than in normal livers (Wicks *et al.*, 1973).

Addition of dexamethazone to the growth medium did not increase the activity of PEPCK in human hepatoma cells, which is in agreement with the findings of Wicks *et al.* (1973) for HTC and RLC cells. In Reuber H35 and $M_1H_1C_1$ cells, however, the activity of PEPCK has been shown to increase 2 - 3 fold in the presence of dexamethazone, dibutyryl cyclic AMP and other cyclic AMP analogues, indicating that during culture these cells retain certain regulatory features of liver whereas HTC and RLC cells do not (Barnett and Wicks, 1971; Wicks and McKibbin, 1972; Wicks *et al.*, 1973; Wagner *et al.*, 1975).

The lack of response of PC, PEPCK and G6Pase to dexamethazone in this hepatoma cell line might suggest a loss of regulatory properties of these enzymes in the cells, which could result from changes during culture or which could be characteristic of the hepatoma from which the cells were derived. However, since little is known concerning the effects of glucocorticoids on human liver PC, PEPCK and G6Pase *in vivo*, it is also possible that conditions were unsuitable for enzyme induction in the hepatoma cells. Activities of PC and G6Pase are elevated in rat livers *in vivo* in response to glucocorticoids (Henning *et al.*, 1963; Prinz and Seubert, 1964; Ashmore and Weber, 1955; Segal and Washko, 1959; Ashmore *et al.*, 1964; Arion and Nordlie, 1967; Nordlie *et al.*, 1965a,1968). Some workers have reported that hepatic PEPCK activity increases in normal rats in response to glucocorticoids (Shrago *et al.*, 1963; Ray *et al.*, 1964; Foster *et al.*, 1966a; Huttner *et al.*, 1974), whilst others have noted such an increase only in diabetic animals (Reshef *et al.*, 1969, 1970; Exton *et al.*, 1973). On the basis of recent studies carried out on rat (Gunn *et al.*, 1975), it has been suggested that glucocorticoids will increase hepatic PEPCK activity only in the absence of insulin and that administration of glucocorticoids results in insulin secretion, thereby actually decreasing the rate of synthesis of the enzyme. However, it seems that the regulation of gluconeogenesis in human liver, in which PEPCK is present in both mitochondrial and cytosol fractions, is different from that postulated for rat liver in which PEPCK is mainly cytosolic (Jomain-Baum and Hanson, 1973). This also appears to be the case in other species such as guinea pig, rabbit and cat in which there is substantial hepatic mitochondrial PEPCK activity (Söling *et al.*, 1970, 1973; Arinze *et al.*, 1973; Johnson *et al.*, 1973; Arinze and Hanson, 1973; Hanson 1974).

The activities of GS b were higher in the rat and human hepatoma cell cultures than in the corresponding tissues, whereas GS a

activities were similar. Total GS activities in the cells were similar to those which have been reported for HeLa cells (Alpers *et al.*, 1963). The increased levels of GS b in the cells can probably be correlated with the presence of glycogen particles which have been detected in the cells (Albrecht *et al.*, 1975; Prozesky, personal communication). As glycogen accumulates in the cells it is possible that, as in liver, a feed back mechanism operates, whereby glycogen synthetase phosphatase is inhibited and the conversion of GS a to GS b is facilitated, so that the effective GS a activity and therefore glycogen synthesis is reduced (Hers *et al.*, 1970; Newsholme and Start, 1973). Bernaert *et al.* (1974) have shown that in cultures of isolated rat hepatocytes, formation of glycogen particles was associated with conversion of GS b to GS a initially and then reconversion of GS a to GS b as glycogen accumulated. It is therefore probable that in the earlier stages of culture, during formation of glycogen, a greater proportion of GS a would have been present in the hepatoma cells. Baba and Tsuiki (1974) have shown that in Ehrlich ascites carcinoma cells there was also a greater proportion of GS b and they found that an 'endogenous factor' which inhibited the conversion of GS b to GS a was present in the cells.

The activities of a number of other enzymes of carbohydrate metabolism, in the rat and human livers, hepatomas and hepatoma cells used in this study, have been measured by others in this laboratory. The levels of the other key gluconeogenic enzyme, fructose-1,6-diphosphatase were found to be lower in hepatomas and hepatoma cells than in livers, consistent with the reduced PC, PEPCK and G6Pase activities and also indicating reduced gluconeogenesis in hepatomas and hepatoma cells. Reduced levels of phosphoglucomutase and increased levels of key glycolytic enzymes indicated reduced glycogenesis and increased glycolysis, respectively, in the hepatomas and hepatoma cells (Balinsky *et al.*, 1972, 1973a; Balinsky,

Cayanis, Mattheyse, unpublished observations).

Significant levels of the gluconeogenic enzymes PC, PEPCK and G6Pase were detected in the livers of human foetuses ranging from 13 to 28 weeks gestational age. Fructose-1,6-diphosphatase activity has also been demonstrated in these tissues (Mattheyse, unpublished observations). These findings are consistent with the observations of Räihä and Lindros (1969) for PC, PEPCK and fructose-1,6-diphosphatase and of Auricchio and Rigillo (1960), Gennser *et al.* (1971) and Schwartz *et al.* (1974) for G6Pase.

The activities of PC, PEPCK and G6Pase in human foetal livers did not appear to show any definite correlation with foetal age. This is possibly because enzyme levels do not increase to adult values until a later stage (perhaps at birth or after) or because of variability resulting from uncontrollable factors, such as length of time between foetal death and abortion or foetal abnormality (even though appearing normal). Auricchio and Rigillo (1960), Gennser *et al.* (1971) and Schwartz *et al.* (1974) also did not find a correlation between G6Pase activities and foetal age.

The presence of PC, PEPCK, G6Pase and fructose-1,6-diphosphatase in human foetal livers suggests that gluconeogenesis could take place at an early stage of development, although at a lower rate than in adult livers, and it seems that the situation resembles that in foetal guinea pig and sheep livers rather than that in foetal rat livers. Significant levels of PC, PEPCK, G6Pase and fructose-1,6-diphosphatase have been detected a few days prior to term in foetal guinea pig livers (Nemeth, 1954; Arinze, 1975; Jones and Ashton, 1976) and in foetal sheep livers at an early stage of development (Warnes *et al.*, 1974; Edwards *et al.*, 1975). It has been confirmed that in guinea pig and sheep there is a capacity for

hepatic gluconeogenesis before birth (Ballard and Oliver, 1965; Arinze, 1975; Jones and Ashton, 1976). In contrast, there is virtually no capacity for gluconeogenesis in foetal rat livers (Ballard and Oliver, 1963, 1965; Philipiddis and Ballard, 1969; Yeung and Oliver, 1967) and the levels of key gluconeogenic enzymes are very low, compared with adult levels, until birth (Dawkins, 1961; Weber and Cantero, 1955b; Burch *et al.*, 1963; Ballard and Hanson, 1967; Greengard, 1969a, 1969b; Chang and Morris, 1973; Greengard and Thorndike, 1974).

In human foetal livers, there was a greater proportion of PEPCK in the mitochondrial fraction (67.1 and 79.2%) than in the cytosol fraction (25.2 and 14.7%), whereas it has been shown that in adult livers, the enzyme is almost equally distributed between the two fractions (Wieland *et al.*, 1968; Brech *et al.*, 1970). In foetal livers of both guinea pig and rat, PEPCK occurs predominantly in the mitochondrial fraction, the proportion of cytosol PEPCK increasing at birth (Ballard and Hanson, 1967; Arinze, 1975; Jones and Ashton, 1976). It appears, however, that in species such as the guinea pig, in which there is a substantial proportion of mitochondrial PEPCK in adult liver, gluconeogenesis is not initiated by the sharp increase in the activity of cytosol PEPCK at birth, as has been suggested for the rat, in which PEPCK is mainly cytosolic in adult liver (Ballard and Hanson, 1967; Arinze, 1975; Jones and Ashton, 1976).

Although there is the capacity for gluconeogenesis in foetal guinea pig and sheep livers and possibly in human foetal livers, it is not known whether this capacity is expressed *in utero*. It has, however, been suggested from studies on lambs that highly oxidised states in foetal livers might limit gluconeogenesis *in utero* (Warnes *et al.*, 1974).

Both a and b forms of GS were found in human foetal livers and the presence of the active a form suggests that glycogen could be synthesised,

although at a low rate, from as early as 13 weeks gestation. This is supported by the fact that significant but relatively low levels of glycogen have been detected in human foetal livers, as well as in livers of foetal sheep and monkeys as early as the second quarter of pregnancy (Villee, 1953; Shelley, 1961; Ballard and Oliver, 1964; Dawkins, 1963a; Gennser *et al.*, 1971). However, in foetal livers of species such as the rat, rabbit and guinea pig, which have a short gestation period, glycogen is virtually absent until the last fifth of pregnancy (Shelley, 1961; Jost, 1960; Kornfeld and Brown, 1962; Ballard and Oliver, 1963; Dawkins, 1963b; Jaquot and Kretchmer, 1964; Burch, 1965; Barnes, 1967; Devos and Hers, 1974). In foetal rat livers the appearance of glycogen is paralleled by the appearance of GS a, although GS b may be present at an earlier stage (Devos and Hers, 1974).

Levels of GS a and GS b were low in the livers of the smaller human foetuses and there was a tendency for both forms to increase up to about 24 weeks, reaching levels higher than adult levels. GS b activity then dropped but GS a activity was not so noticeably affected, perhaps suggesting a slow increase in the rate of glycogen synthesis during development. In foetal livers of all mammalian species studied so far, there is a marked and rapid increase in glycogen levels until just before birth (Barnes, 1968; Gennser *et al.*, 1971; Devos and Hers, 1974). In foetal rat livers, GS a activity increases as glycogen accumulates and then drops just before term when glycogen levels are high (Jaquot and Kretchmer, 1964; Burch, 1965; Schwartz and Rall, 1973; Devos and Hers, 1974). Accumulation of glycogen in foetal rat livers has been shown to be due to a relatively slow rate of synthesis and a virtual absence of degradation (Devos and Hers, 1974).

This study has shown that the rates of gluconeogenesis and glycogen synthesis appear to be reduced in hepatomas, when compared with

normal or host livers. In foetal livers, the rate of gluconeogenesis appears to be lower than in adult livers, whereas the rate of glycogen synthesis seems to be higher. However, relatively little is known concerning the gluconeogenic and glycogenic processes in human tissues and studies of the properties of key enzymes in foetal and adult livers, as well as in hepatomas, would provide valuable information concerning the initiation and regulation of these processes in normal and neoplastic tissue.

CHAPTER II

ISOZYME STUDIES OF NORMAL AND TUMOUR TISSUES AND CELL CULTURES

SUMMARY

1. Starch gel electrophoretic patterns of the isozymes of phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were studied in a number of normal and tumour tissues and cell cultures.

2. Species-specific differences in the mobilities of the various isozymes in rat, human, monkey and bovine tissues and cell cultures were apparent. These differences were of importance in confirming the species origin of cell cultures. It was, however, difficult to distinguish between cells of human and monkey origin unless the human cells had the G6PD B isozyme. Human cells which had the G6PD B isozyme could be distinguished from HeLa cells which had the A isozyme.

3. Species-specific differences in the nature of LDH subunits were apparent from hybridisation studies. Hybridisation of human LDH_5 with pig LDH_1 gave rise to 5 isozymes and hybridisation of human LDH_5 or pig LDH_1 with the LDH isozymes of MDBK cells gave rise to 10 or 11 isozymes in the more cathodal or anodal regions of the gel, respectively.

4. Differences in the isozyme patterns of rat liver and hepatoma were apparent for HK and PK but not for PGM, G6PD or LDH. Liver had HK I,

HK II, HK III and HK IV but whilst HK I was the major liver form, HK II was the major form in hepatoma and also in muscle. HK IV was not detected in hepatoma or muscle. The major liver isozyme PK L was replaced in hepatoma by the minor liver form PK M_2. In two rat hepatoma cell lines the proportion of HK II relative to HK I was increased as compared with hepatoma. HK IV was detected in the cells and PK was present as the M_2 form.

5. The PGM patterns in human hepatoma did not differ from those of liver but in a few hepatomas the G6PD pattern was altered.

6. In human tissues, an additional HK isozyme (II_f) running intermediate between HK II and HK III was detected. This isozyme was observed in foetal muscle, liver, heart and brain, adult liver, hepatoma, meningioma and mesothelioma but not in adult muscle. The only HK isozyme in normal muscle was HK I and normal liver had HK I, which was the major form, HK II_f and HK III. HK IV was detected only in a single host liver specimen obtained during surgery. In addition to HK I, HK II_f and HK III, the isozymes HK IA and HK II were detected in foetal liver. There was a greater proportion of HK II_f in foetal tissues and hepatoma than in normal liver. In hepatoma, HK I usually remained the predominant isozyme but the proportion of HK II increased while that of HK III decreased as compared with host liver. Oesophageal cancer and normal dura had only HK I; this isozyme was also the major form in meningioma and mesothelioma, though these tissues also had traces of HK II and HK II_f. As in rat, an increase in the proportion of HK II relative to that of hepatoma tissue occurred in two human hepatoma cell lines. A similar observation was made with a human oesophageal cancer cell line. The increase of HK II in cell cultures may be related to the high glucose content of the culture medium.

7. The only PK isozyme of human muscle was PK M_1 whereas adult liver contained PK L and a trace of PK M_2. Foetal muscle had PK M_1 and foetal liver had PK L and a trace of PK M_2 as well as another isozyme possibly corresponding to the red cell isozyme of haemopoietic tissue. PK patterns in host livers and hepatomas, while often different from normal liver, were variable and there were no consistent trends. Several host livers and hepatomas had additional strongly-staining bands between PK L and PK M_2. These bands could be L-M_2 hybrids. Meningioma, mesothelioma, human tumour cell lines (hepatoma, oesophageal cancer, HeLa) and fibroblasts had only PK M_2.

8. All five LDH isozymes were detected in human tissues; the patterns in muscle and liver were similar. Tissue-specific differences in isozyme patterns of foetal tissues were not apparent and, as was also the case for HK and PK, there were no age-related differences. The patterns in hepatoma differed from liver patterns in only a few cases when there appeared to be a slight increase in the proportion of H subunits in the tumour tissue. The proportion of H subunits was also increased in the hepatoma cell lines. The patterns in oesophageal cancer, oesophageal cancer cells, dura, mesothelioma and meningioma were similar with LDH_3 and LDH_4 as the major forms.

9. Clear protein bands were obtained on polyacrylamide disc gel isoelectric focusing but isozyme bands were not as well-defined. Differences between liver and hepatoma patterns were apparent for total protein, HK and LDH.

METHODS

Chemicals

Substrates, cofactors and enzymes

Monosodium phosphoenolpyruvate (PEP), disodium ATP, disodium ADP, disodium NADP, disodium NADH, disodium AMP, tetracyclohexylammonium fructose-1,6-diphosphate (FDP) and the enzymes glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK) and lactate dehydrogenase (LDH) were obtained from Biochemica Boehringer, Mannheim, Germany; NAD was from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; potassium pyruvate from Calbiochem, Los Angeles, California, U.S.A. and lactic acid, glucose-1-phosphate (G1P) and disodium glucose-6-phosphate (G6P) from British Drug Houses, Poole, England.

Other chemicals

Dithiothreitol (DTT) was obtained from Calbiochem; phenazine methosulphate (PMS) from Sigma and 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-nitrophenyl tetrazolium salt (INT) from National Biochemical Corp., Cleveland, Ohio, U.S.A. Connaught hydrolysed starch was from Connaught Medical Research Laboratory, Toronto, Canada; Bacto-Agar from Difco Laboratories, Detroit, Michigan, U.S.A.; acrylamide, NN'-methylenebisacrylamide and riboflavin from British Drug Houses; Coomassie Brilliant Blue (R250) from Imperial Chemical Industries, Johannesburg, South Africa and Ampholines from LKB, Uppsala, Sweden. Other standard chemicals were obtained from British Drug Houses, Poole, England, E. Merck, Darmstadt, Germany and Hopkin and Williams, Chadwell Heath, England, and were of analytical grade.

Tissues and Cell Cultures

Rat and human livers and hepatomas were obtained from the sources described in Chapter I (p. 80). Muscle was obtained from normal rats and normal human muscle was obtained at autopsy within six hours of death. Human oesophageal cancer and meningioma tissues were obtained during surgery and mesothelioma at autopsy. Livers of vervet monkey and chacma baboon were supplied by Dr. P.A.D. Winter. Tissues were kept on ice if electrophoresis was to be carried out immediately, otherwise they were stored at -20° until required. Rat and human hepatoma cells were from the sources described previously (p. 81). In addition, human hepatoma cells and oesophageal cancer cells were provided by Dr. J.J. Alexander. Other cell cultures, HeLa, human fibroblasts, VERO (vervet kidney) and MDBK (bovine kidney) used for comparative purposes were supplied by Prof. O.W. Prozesky and Dr. J.M. Whitcutt. Cells were obtained in serum-free medium after trypsinisation. They were centrifuged at 50 g, at room temperature, for 2 minutes, washed twice with 0.9% saline and extracts were prepared immediately.

Starch gel electrophoresis

Preparation of extracts

Tissue extracts were prepared at 0°, either in the homogenising medium described by Shonk and Boxer (1964), which contained 150 mM potassium chloride, 50 mM potassium bicarbonate, 6 mM EDTA and 1 mM DTT, or in 10 mM Tris-HCl, pH 7.4 containing 1 mM DTT. An Ultra-Turrax homogeniser was used.

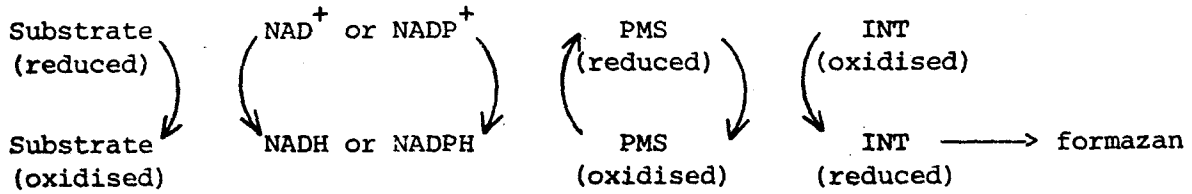
In all cases, except for LDH, 1:1 homogenates were prepared. For LDH, 1:10 homogenates were used. The homogenates were centrifuged at

36,000 g, 4°, for 30 minutes, using an MSE 18 High Speed refrigerated centrifuge.

Cell extracts were prepared either in 50 mM Tris-HCl, pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and 5 mM mercaptoethanol or in 10 mM Tris-HCl, pH 7.4 containing 1 mM DTT, by freezing and thawing three times in an acetone-dry ice mixture. The extracts were centrifuged at 36,000 g, 4°, for 1 hour, using an MSE 18 High Speed refrigerated centrifuge. Extracts were kept on ice if electrophoresis was to be carried out immediately, otherwise they were stored at -20° until required.

General method

Starch was boiled with the appropriate gel buffer (24 g starch per 200 ml buffer for each gel). The solution was degassed, poured into frames (18.5 cm x 10 cm x 0.5 cm) and allowed to set. Samples were applied to slits in the gels using Whatman 3 MM paper. Shandon electrophoresis tanks were used with 300 - 400 ml buffer in each electrode compartment. Three layers of Whatman 3 MM paper formed each wick, the gels were covered with polythene and electrophoresis was carried out in the cold room. After electrophoresis, isozymes were detected on the sliced gels by reactions coupled to reduction of NAD^+ or $NADP^+$, PMS and INT, with subsequent formation of a red formazan precipitate in the regions of activity.



Stains were prepared in 0.83% agar. The agar was boiled in stain buffer for about 30 minutes, cooled to 40° and the required substances added. The mixture was then poured onto the starch gel and the gels were developed

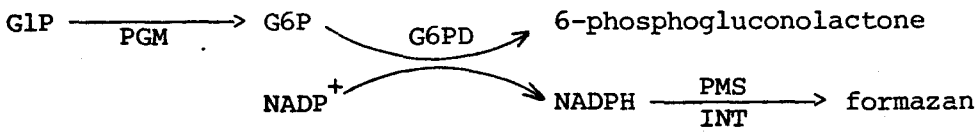
in the dark.

Specific buffers and stains

Phosphoglucomutase (PGM)

Buffers were prepared as described by Shaw and Koen (1968). The gel buffer was a 1 in 10 dilution of the electrode buffer which was 100 mM Tris - 100 mM maleic acid - 10 mM EDTA - 10 mM magnesium chloride, pH 7.4. Electrophoresis was carried out for 17 hours at a constant voltage of 9.2 volts/cm gel length.

The stain was based on the following reactions:-

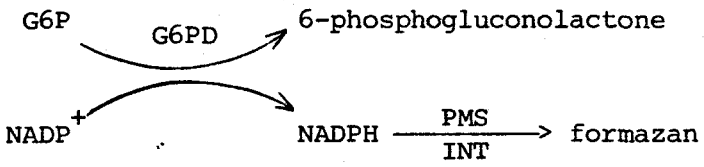


The stain buffer, 50 mM Tris-HCl, pH 8.0 contained 30 mM magnesium chloride, 3 mM $NADP^+$, 3.4 mM glucose-1-phosphate, 0.5 mg INT/ml, 0.025 mg PMS/ml, 0.35 U glucose-6-phosphate dehydrogenase/ml and 0.83% agar. Glucose-1-phosphate was omitted from the blank.

Glucose-6-phosphate dehydrogenase (G6PD)

Electrophoresis was carried out as described by Shaw and Koen (1968). The gel buffer was 50 mM Tris - 1.6 mM EDTA - 97 mM boric acid, pH 8.0 and 0.02 mM NADP was added to the hot starch solution before degassing. The electrode buffer was 500 mM Tris - 16 mM EDTA - 647 mM boric acid, pH 8.0. Electrophoresis was carried out for 17 hours at a constant voltage of 8.1 volts/cm gel length.

The stain was based on the following reactions:-

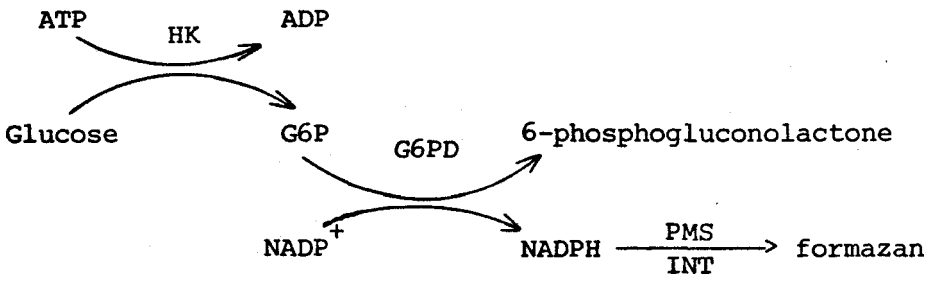


The stain buffer, 50 mM Tris-HCl, pH 7.0 contained 50 mM glucose-6-phosphate, 0.8 mM $NADP^+$, 0.5 mg INT/ml, 0.02 mg PMS/ml and 0.83% agar. Glucose-6-phosphate was omitted from the blank.

Hexokinase (HK)

The method was similar to that of Katzen and Schimke (1965). The gel buffer was 20 mM sodium diethylbarbiturate (barbitone sodium) - 4 mM diethylbarbituric acid (barbitone), pH 8.6, containing 2.5 mM EDTA and 0.1 mM DTT. The electrode buffer was the same as the gel buffer. Electrophoresis was carried out for 17 hours at a constant current of 1.1 - 1.2 mA/cm gel width. In later studies, where indicated, the current was increased to 1.5 - 1.8 mA/cm gel width.

The stain was based on the following reactions:-



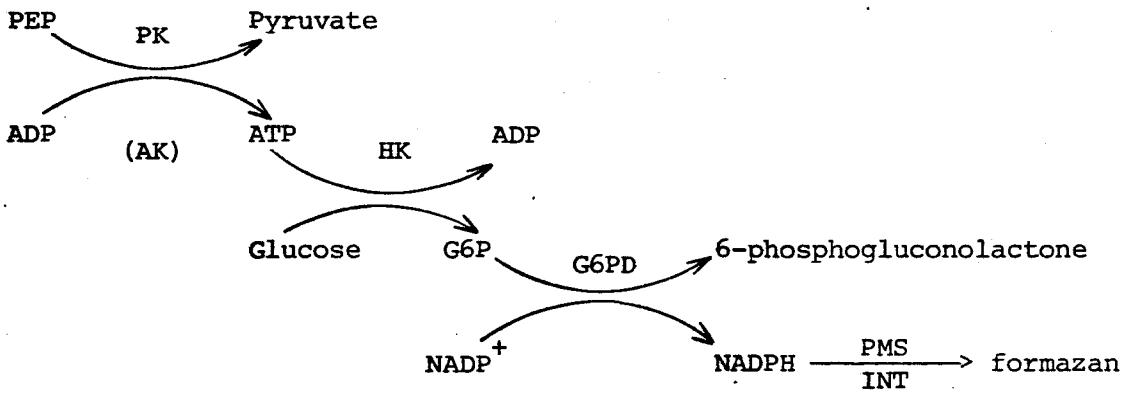
The stain buffer, 100 mM Tris-HCl, pH 7.4 contained 0.75 mM $NADP^+$, 10 mM magnesium chloride, 2 mM potassium cyanide, 3 mM ATP, glucose 0.5 mM (to stain for low K_m (glucose) isozymes I, II and III) or 0.1 M (to stain for the high K_m (glucose) isozyme HK IV), 0.3 U glucose-6-phosphate dehydrogenase/ml, 2 mg INT/ml, 0.04 mg PMS/ml and 0.83% agar. Blanks were prepared in which ATP was omitted and 0.1 M glucose was present or in which

ATP was present and glucose was omitted.

Pyruvate Kinase (PK)

The gel buffer was 20 mM sodium diethylbarbiturate (barbitone sodium) - 4 mM diethylbarbituric acid (barbitone), pH 8.6 containing 2.5 mM EDTA and 0.1 mM DTT. The electrode buffer was the same as the gel buffer and in later experiments, where indicated, 0.1 mM fructose-1,6-diphosphate was included in both buffers. Electrophoresis was carried out for 17 hours at a constant current of 1.1 - 1.2 mA/cm gel width. At a later stage, where indicated, the current was increased to 1.6 - 1.8 mA/cm gel width and the M_1 and M_2 isozymes were separated.

The stain, which was a modification of that described by Fildes and Harris (1966) for adenylate kinase, was based on the following reactions:-



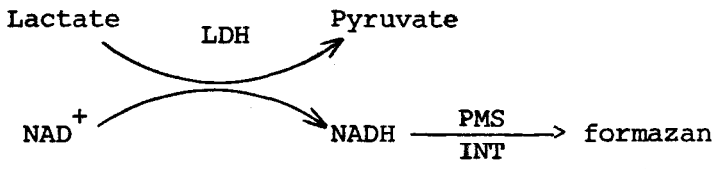
The stain buffer, 100 mM Tris-HCl, pH 7.4, contained 37 mM magnesium chloride, 0.74 mM $NADP^+$, 2 mM ADP, 5.3 mM phosphoenolpyruvate, 15 mM glucose, 20 mM AMP (to inhibit AK), 0.5 U hexokinase/ml, 0.5 U glucose-6-phosphate dehydrogenase/ml, 3.7 mg INT/ml, 0.074 mg PMS/ml and 0.83% agar. Phosphoenolpyruvate was omitted from the blank.

Lactate dehydrogenase (LDH)

To prevent sub-band formation, 1 mM NAD was added to the tissue

or cell extracts. The gel buffer was 76 mM Tris - 5 mM citric acid, pH 8.6 and 0.04 mM NAD was added to the starch solution before degassing. The electrode buffer was 300 mM boric acid - sodium hydroxide, pH 8.0. Electrophoresis was carried out for 3½ hours at a constant current of 2 mA/cm gel width. Addition of 0.25% albumin and 10% globulin to the homogenates before electrophoresis did not affect the isozyme patterns.

The stain was prepared essentially according to Shaw and Koen (1968) and was based on the following reactions:-



The stain buffer, 50 mM Tris - HCl, pH 7.0, contained 0.1 mM NAD^+, 220 mM sodium lactate, 0.35 mg INT/ml, 0.02 mg PMS/ml and 0.83% agar. Sodium lactate was omitted from the blank.

Apparent Michaelis constants for phosphoglucomutase (PGM)

Rat and human liver and hepatoma extracts were prepared in homogenising medium (as described on p.113) using an Ultra-Turrax homogeniser. The homogenates were centrifuged at 36,000 g, 4°, for 30 minutes, using an MSE 18 High Speed refrigerated centrifuge.

PGM activity in the supernatants was determined using the continuous spectrophotometric method described by Shonk and Boxer (1964). The assay was based on the reactions shown below and the formation of NADPH was followed at 340 nm using a Unicam SP 800 spectrophotometer.

$$\text{G1P} \xrightarrow{\text{PGM}} \text{G6P}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

The reaction mixture contained 50 mM triethanolamine - 5 mM EDTA - 10 mM magnesium chloride, pH 7.4, 0.36 mM $NADP^+$, 4 U glucose-6-phosphate dehydrogenase, glucose-1-phosphate and enzyme in a total volume of 3 ml. Glucose-1-phosphate concentrations were varied from 0.33 to 2 mM or from 0.5 to 3 mM. The mixture was preincubated at 30° for 10 minutes in the absence of glucose-1-phosphate to allow for activation of the enzyme by magnesium. The reaction was then initiated by addition of glucose-1-phosphate. Glucose-1-phosphate was omitted from the blank.

Reciprocal velocities were plotted against reciprocal substrate concentrations (Lineweaver and Burk, 1934) and Michaelis constants and their standard errors were determined as described on p.184 using the computer programme of Cleland (1963).

Lactate dehydrogenase (LDH) hybridisation studies

The LDH_5 isozyme of a human host liver was separated from the other four LDH isozymes by means of a batchwise procedure using DEAE-cellulose. The liver was homogenised in the homogenising medium described on p.113, using an Ultra-Turrax homogeniser. After centrifugation at 36,000 g, 4°, for 30 minutes, using an MSE 18 High Speed refrigerated centrifuge, the supernatant was put onto DEAE-cellulose which had been equilibrated with 8 mM sodium phosphate buffer, pH 7.0. After 15 minutes, the extract was sucked off and LDH_5 was obtained by washing the DEAE-cellulose with 8 mM sodium phosphate buffer, pH 7.0. The human liver LDH_5 was then hybridised with pig heart LDH_1 (obtained from Boehringer) using the method described by Chilson *et al.* (1965). A mixture of the two isozymes was prepared in the

ratio of 1:1 with respect to enzyme activity. An equal volume of 0.2 M potassium phosphate, pH 7.0 containing 1 M sodium chloride was added to the mixture. After freezing quickly in an acetone-dry ice bath, the mixture was allowed to thaw slowly at room temperature. Fast freezing and slow thawing was carried out 2 or 3 times. Reactivation was carried out by allowing the mixture to stand at room temperature for 30 minutes and then dialysing for one hour at 4° against distilled water containing 0.1 mM DTT.

The human liver LDH_5 and pig heart LDH_1 were also hybridised with the LDH isozymes of bovine kidney cells. The LDH isozymes of bovine kidney cells were extracted in 10 mM Tris-HCl, pH 7.4 containing 1 mM DTT, by freezing and thawing twice in an acetone-dry ice mixture. The extracts were centrifuged at 36,000 g, 4°, for 20 minutes, using an MSE 18 High Speed refrigerated centrifuge and the clear supernatant was used for hybridisation studies.

Hybrid isozyme patterns were examined by starch gel electrophoresis, as described above (p.117).

Lactate dehydrogenase activities in the extracts and hybrids were determined using a continuous spectrophotometric method, whereby formation of NAD^{+} was followed at 340 nm using a Unicam SP 800 spectrophotometer (Shonk and Boxer, 1964).

$$\text{pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{lactate} + NAD^{+}$$

The assay mixture contained 50 mM triethanolamine - 5 mM EDTA, pH 7.4, 0.15 mM NADH, 2.9 mM potassium pyruvate and 0.1 ml of suitably diluted enzyme in a volume of 3 ml. The reaction was initiated by addition of enzyme and potassium pyruvate was omitted from the blank.

Isoelectric focusing

Tissue extracts were prepared in 10 mM Tris-HCl, pH 7.4 containing 1 mM DTT using an Ultra-Turrax homogeniser. The homogenates were centrifuged at 36,000 g, 4°, for 20 minutes, using an MSE 18 High Speed refrigerated centrifuge. Soft gels were obtained if homogenising medium (as on p.113) containing potassium chloride and potassium bicarbonate was used.

The sample was incorporated into the gel mixture before polymerisation. The gel mixture was prepared according to the method of Smith *et al.* (1971) and contained (for 1 gel) 0.6 ml of a solution of 30% acrylamide and 1% NN´-methylenebisacrylamide, 0.16 ml of a 0.014% riboflavin solution, 0.06 ml Ampholine solution (40%), 0.15 ml tissue extract and water to give a volume of 2.4 ml. All solutions were made up using distilled degassed water.

The Shandon disc gel electrophoresis apparatus was used and the gel mixture was introduced into 65 mm running tubes, using a syringe and polyethylene tubing to avoid formation of bubbles. Photopolymerisation took place under an ultra-violet lamp for 1½ - 2 hours. The tubes were inserted in the electrophoresis apparatus which had 250 ml 0.2% H_2SO_4 in the anode compartment and 300 ml 0.4% ethanolamine in the cathode compartment.

Isoelectric focusing was carried out in the cold room using a constant voltage of 30 volts/tube. The focusing time was 2 hours for Ampholines in the pH range 3 - 10 and three hours for Ampholines in the pH range 5 - 8. After isoelectric focusing, the gels were removed from the running tubes using a syringe and distilled water and were stained using either the protein stain described below or the specific enzyme

stains which were as described for starch gel electrophoresis, except that the agar was omitted and the gel immersed in liquid stain.

The pH gradient was determined by cutting an unstained gel into small equal sections and placing each section in 1 ml boiled out water for 1 hour and then measuring the pH of the solution.

Protein stain for polyacrylamide gels after isoelectric focusing

The method was that of Malik and Berrie (1972) and did not necessitate removal of Ampholines prior to staining.

100 ml 2% aqueous Coomassie Brilliant Blue was added to 100 ml 2N sulphuric acid. The precipitate was left to sediment for a while and the green supernatant solution was filtered. 10 N potassium hydroxide was added until the liquid took on a bluish tint and 10 g trichloroacetic acid was then added per 100 ml.

ISOZYME NOMENCLATURE

The glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) isozymes, separated on starch gel electrophoresis, are designated as follows:-

The faster moving human G6PD isozyme corresponding to the A phenotype is G6PD A and the slower moving form corresponding to the B phenotype is G6PD B (Boyer *et al.*, 1962).

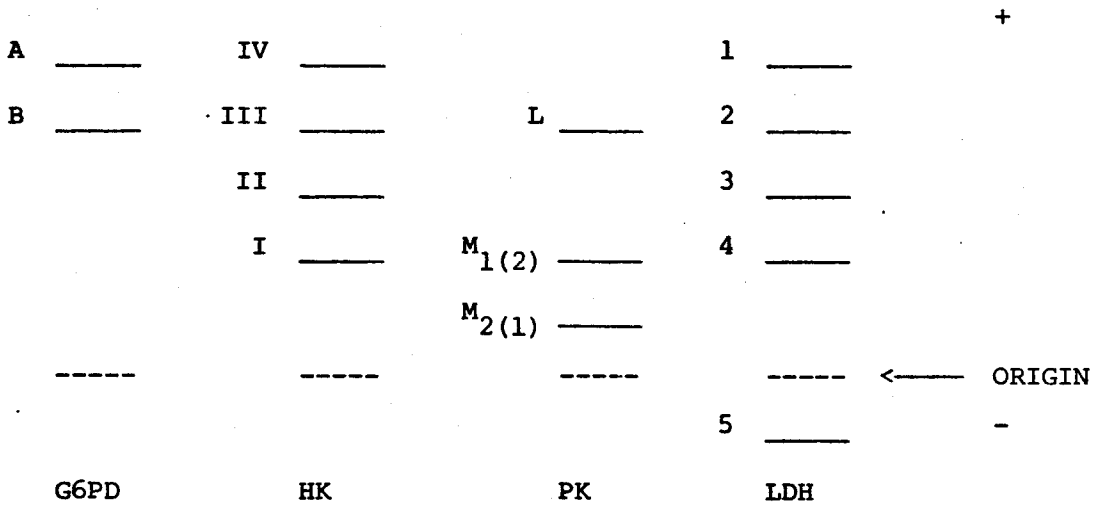
The HK isozymes are referred to as I, II, III and IV in order of increasing mobility towards the anode (Katzen and Schimke, 1965).

The major liver form of PK is referred to as PK L, the minor

liver form as PK M_2 and the muscle form as PK M_1 (Imamura and Tanaka, 1972). The relative mobilities of PK M_1 and PK M_2 vary, depending on the species concerned and on whether or not fructose-1,6-diphosphate is included in the electrophoresis buffers. In cases where the separation of the M_1 and M_2 forms was unclear and it was not certain which was present, the isozyme is referred to as PK M.

The LDH isozyme migrating towards the cathode is LDH_5 and the isozymes LDH_4, LDH_3, LDH_2 and LDH_1 migrating towards the anode are numbered in order of increasing mobility (Markert and Møller, 1959).

The relative mobilities of the various isozymes are shown below.



RESULTS

ISOZYME PATTERNS ON STARCH GEL ELECTROPHORESIS

The isozyme patterns of phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were compared in a variety of tissues and cell cultures. These included adult rat and human muscle, liver and hepatomas, human foetal tissues, other human tumours (oesophageal cancer, meningioma, mesothelioma) and cell cultures of tumours and fibroblasts.

Relative mobilities of isozymes in tissues of various species

Extracts prepared from tissues of various species were run alongside one another on the same gels and the mobilities of the various isozymes were compared. The relative mobilities of the G6PD, HK, PK and LDH isozymes in rat, human, vervet monkey and chacma baboon livers are shown in Fig. 1.

The major G6PD isozyme in livers of rat, monkey and baboon ran very similarly to human G6PD A.

Comparison of HK and PK isozyme patterns in rat and human muscle, livers and hepatomas showed that in rat, the HK isozymes I, II and II were slower moving and the PK isozymes L, M_1 and M_2 faster moving than in human. In monkey and baboon livers, the mobilities of the HK isozymes I, II and II and PK isozymes L and M were similar to those of the corresponding human forms.

With the exception of LDH_5, which had a slightly greater mobility towards the cathode, the LDH isozymes of rat muscle, liver and hepatoma had similar mobilities to the corresponding human forms. Mobilities of all five LDH isozymes in monkey and baboon livers were indistinguishable

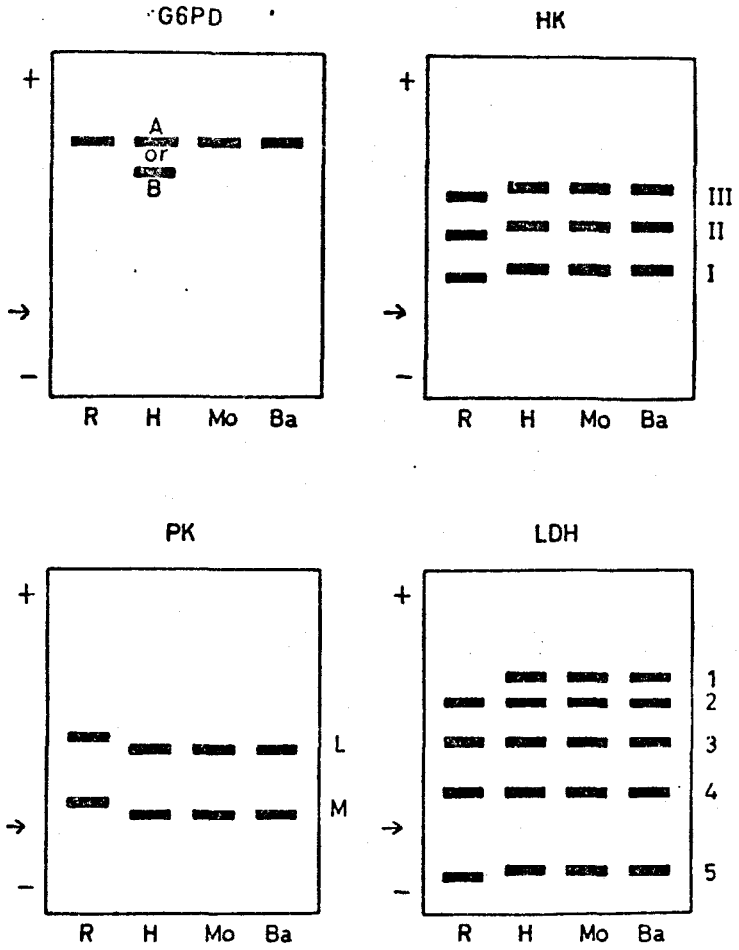


FIGURE 1. Relative mobilities of glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) isozymes in livers of different species; R - rat, H - human, Mo - vervet monkey, Ba - chacma baboon. The origin is indicated by an arrow.

from human.

Species origin of cell cultures

Species differences in isozyme mobilities provide a means of checking that cells have not been contaminated during culture. This was shown when isozyme patterns in what were thought to be human hepatoma cells were compared with human tissue patterns and differences in isozyme mobilities were observed. There were two bands of G6PD activity in the cells, the slower one being similar to human G6PD B and the faster one being slower than human G6PD A. In gels stained using 0.5 mM glucose two HK isozymes were present, the slower one corresponding to human HK I and the other being slightly faster than human HK II. HK IV was also detected in the cells when 0.1 M glucose was included in the stain but was not observed in human tissues. The mobility of the PK isozyme present in the cells was intermediate between human PK M and PK L. The LDH isozymes in the cells were closer together than the human isozymes. The differences in LDH isozyme mobilities were clearly shown when mixtures of cell and human host liver or hepatoma extracts were used and the isozymes present in the individual extracts were separated in the mixtures. The cells were obviously not of human origin and it was suspected that during culture they could have been contaminated with bovine kidney (MDBK) cells, which were growing in the same laboratory. The mobilities of the G6PD, HK, PK and LDH isozymes present in the cells corresponded exactly with those of bovine tissues and the patterns were identical to those of MDBK cells, confirming that the "human hepatoma" cells were in fact MDBK cells.

It was thus necessary to ensure, as far as possible, that other rat and human cells used in this study were not contaminated. The G6PD, HK, PK and LDH isozyme patterns in the cells were compared with those in tissues of the respective species as well as with those in

other cell cultures, which were growing in the same laboratories and which could have been possible contaminants.

Two rat hepatoma cell lines, two human hepatoma cell lines, a human oesophageal cancer cell line, HeLa cells, a human fibroblast cell line, vervet monkey kidney (VERO) and bovine kidney (MDBK) cells were examined. The patterns in the two rat hepatoma cell lines were similar and no distinction is made between them. In this and subsequent sections, the human hepatoma cells provided by Prof. Prozesky are referred to as hepatoma cells (P) and those provided by Dr. Alexander are referred to as hepatoma cells (A).

Comparison of cell patterns with tissue patterns showed that, although there were differences in isozyme distribution, the mobilities of the G6PD, HK, PK and LDH isozymes present in the cells did in fact correspond with the mobilities of forms present in tissues of the species of origin.

The relative mobilities of the G6PD, HK, PK and LDH isozymes in rat hepatoma cells, human hepatoma cells (P) and also in cells of monkey (VERO) or bovine (MDBK) origin are shown diagrammatically in Fig. 2. The species-specific differences in the mobilities of the HK, PK and LDH isozymes present in the cell cultures easily distinguished human cells from cells of rat or bovine origin. The HK, PK and LDH isozyme mobilities were very similar in monkey cells and human cells and the G6PD isozyme in monkey cells ran similarly to human G6PD A. In human hepatoma cells (P) and in human oesophageal cancer cells the G6PD B isozyme was present, thus distinguishing these cells from cells of monkey origin. However, the G6PD A isozyme was present in human hepatoma cells (A) and it was therefore not possible to distinguish electrophoretically between these cells and monkey cells.

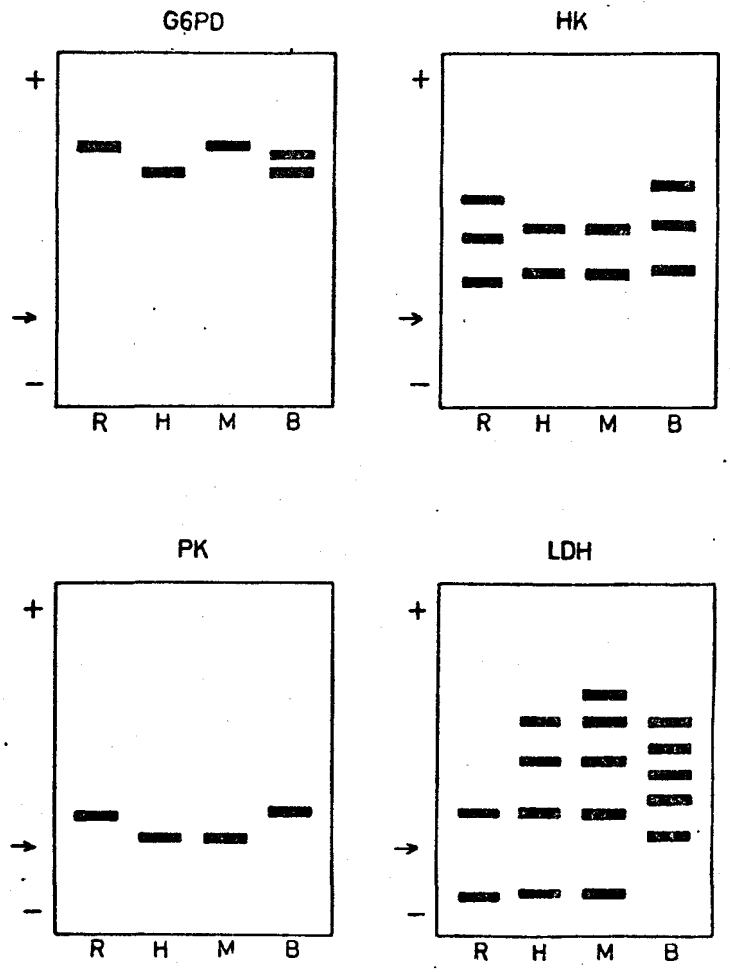


FIGURE 2. Relative mobilities of glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) isozymes in cell cultures derived from different species; R - rat hepatoma, H - human hepatoma (P), M - vervet monkey kidney (VERO), B - bovine kidney (MDBK). The origin is indicated by an arrow.

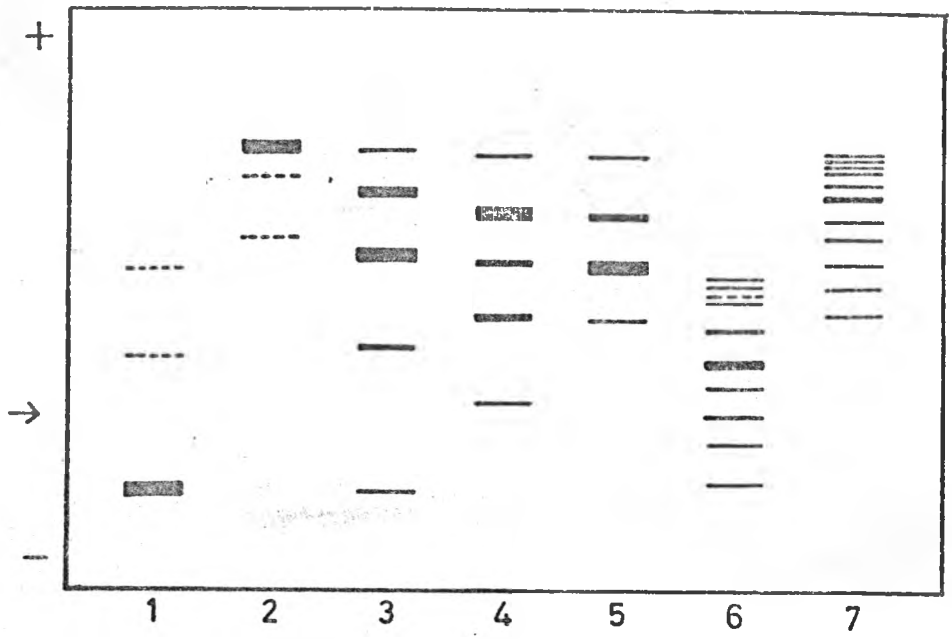
Except for human fibroblasts which had only HK I, the cell cultures studied all had HK I and HK II and in addition, rat hepatoma and MDBK cells had HK IV. The cell cultures all had PK M. Rat hepatoma cells often had only LDH_5 but LDH_4 was also detected occasionally. All five LDH isozymes were present in the other cell cultures, except for human hepatoma cells (P) and HeLa cells which did not have LDH_1.

Species differences in the nature of lactate dehydrogenase (LDH) subunits - hybridisation studies

The isozyme patterns obtained when human LDH_5 was hybridised with pig LDH_1 and when the LDH isozymes of MDBK cells were hybridised alone and with human LDH_5 or pig LDH_1 are shown in Fig. 3.

Five bands were obtained when LDH_5 from human host liver was hybridised with pig heart LDH_1. The faintest most cathodic band had the same mobility as human LDH_5 and the most anodic the same mobility as pig LDH_1. The LDH_3 hybrid isozyme had the greatest intensity and was intermediate between pig LDH_3 and human LDH_3. The pattern was the same whether the mixture was frozen and thawed two or three times during hybridisation. When reactivation was carried out in the presence of 0.012 mM NADH, there was no change in enzyme activity or isozyme pattern but dialysis of reactivated mixtures overnight resulted in a loss of the LDH_4 and LDH_5 isozymes.

Hybridisation of the LDH isozymes of MDBK cells alone resulted in a different intensity distribution of the bands as shown (Fig. 3). When the LDH isozymes of MDBK cells were hybridised with human LDH_5 there were at least ten bands in the more cathodic region of the gel and when they were hybridised with pig LDH_1 there were also ten or eleven bands,



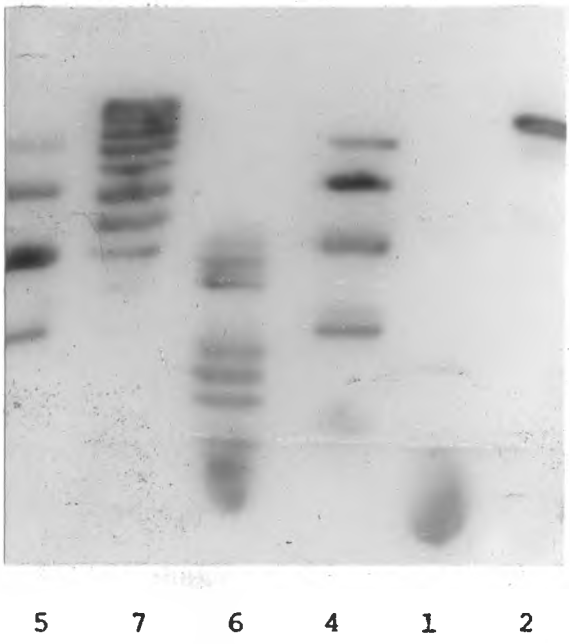


FIGURE 3. Lactate dehydrogenase (LDH) hybrid isozyme patterns; 1 - human host liver LDH_5, 2 - pig heart LDH_1, 3 - human LDH_5 hybridised with pig LDH_1, 4 - LDH isozymes of bovine kidney cells (MDBK), 5 - LDH isozymes of MDBK cells hybridised alone, 6 - human LDH_5 hybridised with MDBK cell isozymes, 7 - pig LDH_1 hybridised with MDBK cell isozymes.

but the pattern was shifted more towards the anode.

Isozyme patterns in rat tissues and cell cultures

The PGM, G6PD, HK, PK and LDH isozyme patterns in rat muscle, liver, hepatoma and hepatoma cells are shown diagrammatically in Fig. 4 and some examples are given in Fig. 5.

There was hardly any variation in the patterns in tissues derived from several different individuals and when the patterns in cell cultures were examined on a number of occasions, they were found to be the same.

There were no apparent differences between the patterns in extracts prepared from fresh tissue or from tissue which had been stored at -20° for up to one month, or between the patterns in freshly prepared cell extracts and cell extracts which had been stored at -20° for up to one month.

Three PGM isozymes were detected in the tissues and cells and there were no apparent differences between the patterns in muscle, liver, hepatoma and hepatoma cells.

The apparent Michaelis constants of normal liver, host liver and hepatoma PGM for glucose-1-phosphate were found to be 1.83 ± 0.03, 1.66 ± 0.04 and 1.56 ± 0.05 mM, respectively.

There were two bands of G6PD activity in muscle, a major band and a fainter slower-moving band. The major isozyme in normal liver, host liver and hepatoma, which was frequently split into two sub-bands, was slightly faster than the major muscle form. In one instance, a slower band, not present in other tissues, was detected in a host liver extract

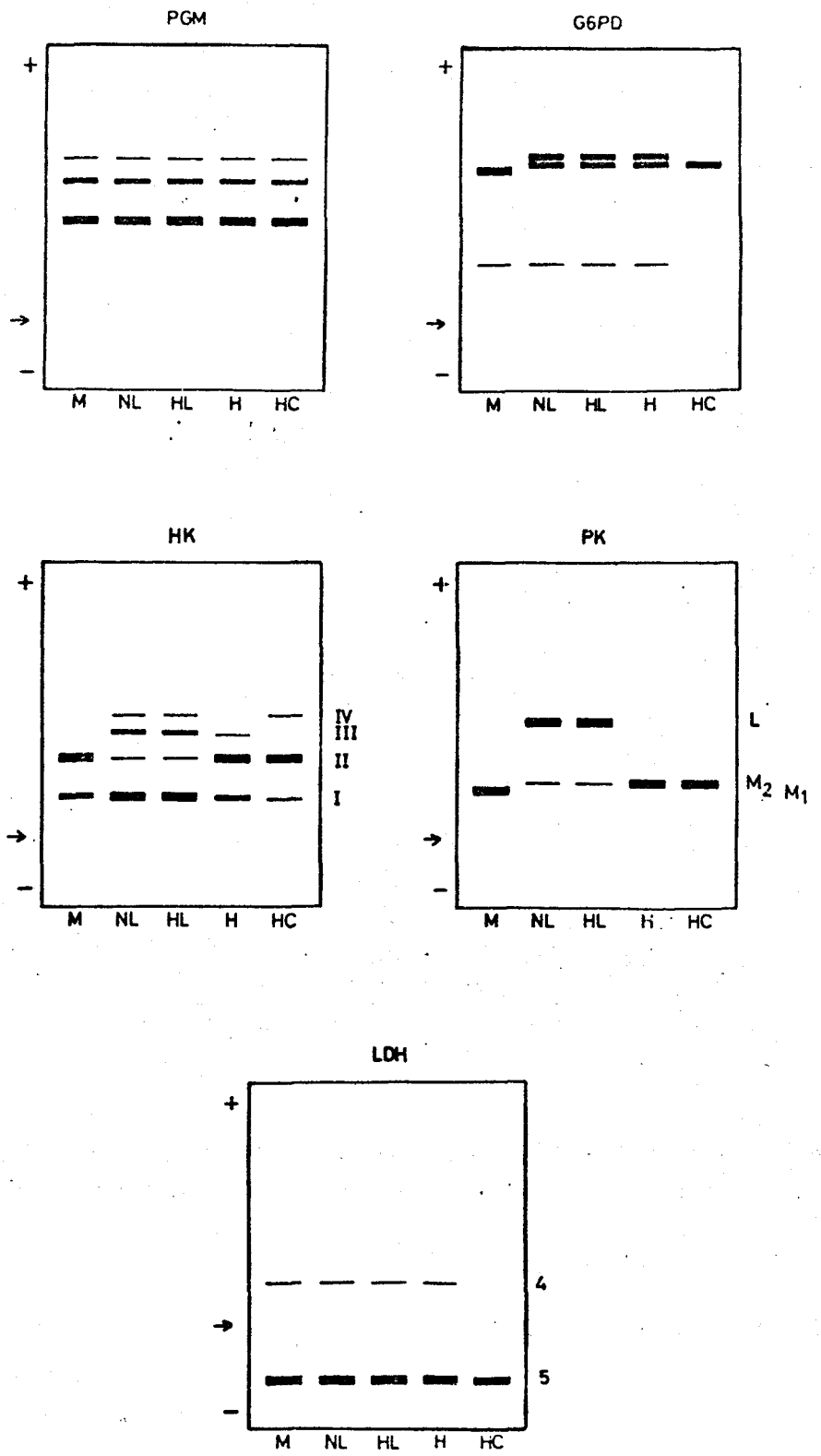


FIGURE 4. Diagrammatic representation of phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) isozyme patterns in rat muscle (M), normal liver (NL), host liver (HL), hepatoma (H) and hepatoma cells (HC). The origin is indicated by an arrow.

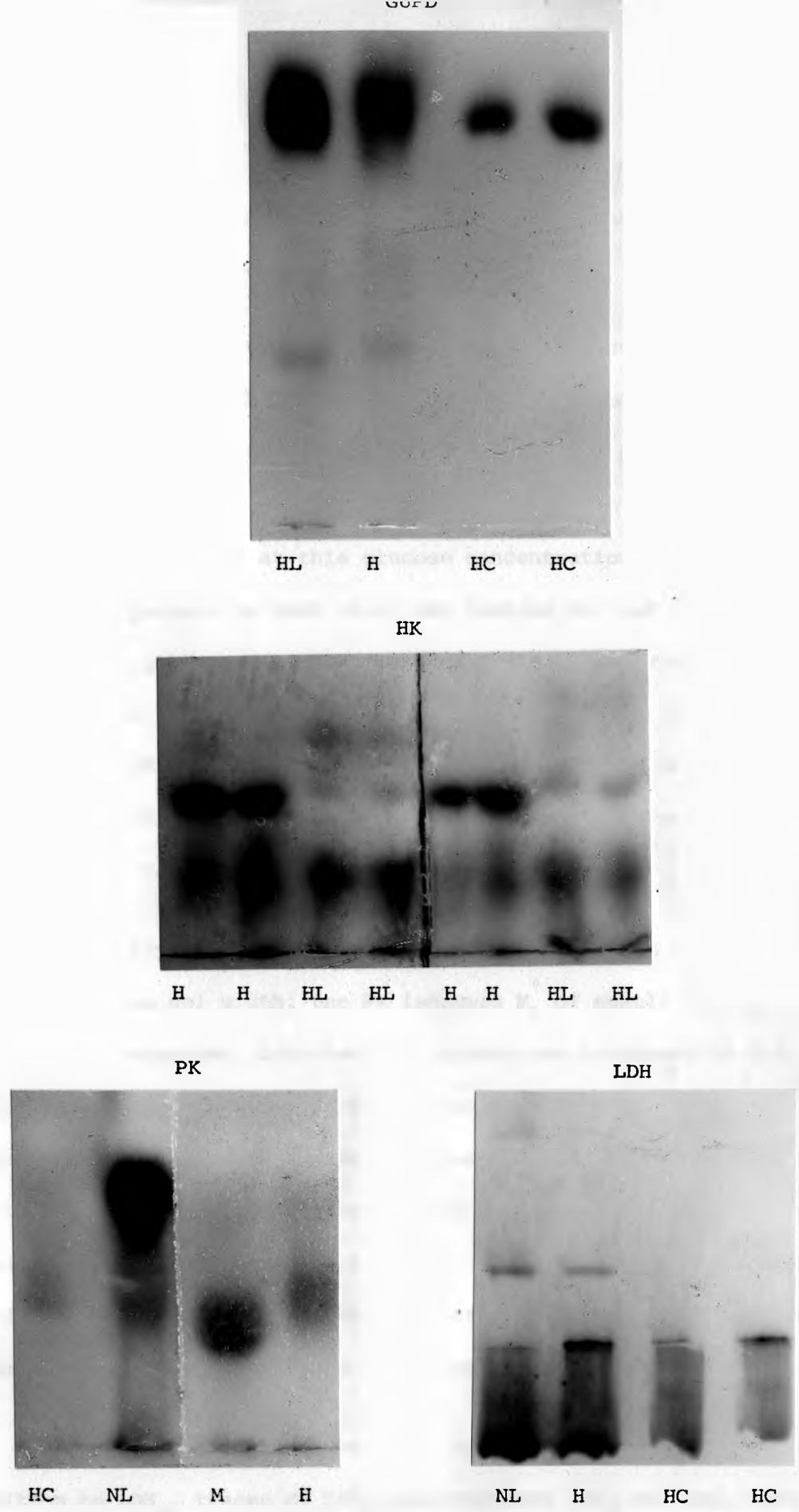


FIGURE 5. Glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) isozyme patterns in rat host liver (HL), hepatoma (H), hepatoma cells (HC), normal liver (NL) and muscle (M). For HK, the gel on the left of the photograph was stained with 0.5 mM glucose and that on the right with 0.1 M glucose.

(this liver had, however, been stored frozen for over a year). In the hepatoma cells there was a single isozyme corresponding to the slower sub-band of the major liver and hepatoma isozymes.

Hexokinase isozymes were separated using a current of 1.1 - 1.2 mA/cm gel width. Muscle had HK II, which was the major form, and HK I. In normal liver HK I was the predominant isozyme, and HK II, HK III and HK IV were detected. HK IV was only detected when 0.1 M glucose was included in the stain and at this glucose concentration HK III was somewhat inhibited. The pattern in host liver was similar to that of normal liver, and the hepatoma pattern resembled that of muscle. Hexokinases I, II and III were found in hepatoma and HK II was the major form. In hepatoma cells, HK I and HK II were present but the proportion of HK II was increased with respect to HK I as compared with hepatoma tissue or muscle. HK IV was also detected in hepatoma cells.

Initially, when electrophoresis was carried out using a current of 1.1 - 1.2 mA/cm gel width, the PK isozymes M_1 of muscle and M_2 of liver were not separated, but when the current was increased to 1.6 - 1.8 mA/cm gel width the two forms were distinguishable. The gel and electrode buffers contained 0.1 mM fructose-1,6-diphosphate and the mobility of PK M_2 was greater than that of PK M_1. In muscle only the M_1 isozyme was present. In both normal and host liver, PK L was the major form and a trace of PK M_2 was detected, whereas in hepatoma and hepatoma cells there was only the M_2 isozyme.

The predominant LDH isozyme in tissues and cell cultures was LDH_5. In addition to LDH_5, traces of LDH_4 and sometimes LDH_3 and LDH_2 were detected in muscle, normal and host liver and hepatoma. Generally, there was only LDH_5 in hepatoma cells, but on occasions LDH_4 and LDH_3 were detected in both the lines.

Isozyme patterns in human tissues and cell cultures

Phosphoglucomutase (PGM) and glucose-6-phosphate dehydrogenase (G6PD)

Multiple forms of PGM were detected in muscle, liver and hepatoma and the patterns varied in tissues derived from different individuals, according to the phenotype. There were no apparent differences in the patterns in different tissues derived from the same individual. The patterns obtained for three different host livers and their corresponding hepatomas are shown in Fig. 6.

The apparent Michaelis constants of host liver and hepatoma PGM for glucose-1-phosphate were found to be 1.46 ± 0.04 and 1.38 ± 0.03 mM, respectively.

With only a few exceptions, the G6PD isozyme patterns were the same in tissues derived from the same individual and usually corresponded to either the A or B phenotype. In general, hepatoma patterns were similar to host liver patterns, but in two hepatomas (both of which had been frozen for over a year) there was a much faster-moving band and very little of the G6PD B isozyme, which was the form present in the corresponding host livers. In one case, in the surgical host liver and hepatoma, a different G6PD variant was detected and as shown in Fig. 7, two bands of G6PD activity were apparent in both tissues, the slower moving band was similar to G6PD B and the faster band was slightly slower than G6PD A. In host liver, however, the intensity of the slow band was much greater than that of the fast band, whereas in hepatoma the pattern was reversed.

As shown in Fig. 7, the G6PD isozyme in hepatoma cells (P) and in the oesophageal cancer cells corresponded to the B phenotype and thus distinguished these cells from other cells of human origin which were growing in the same laboratory, namely HeLa cells and fibroblasts which

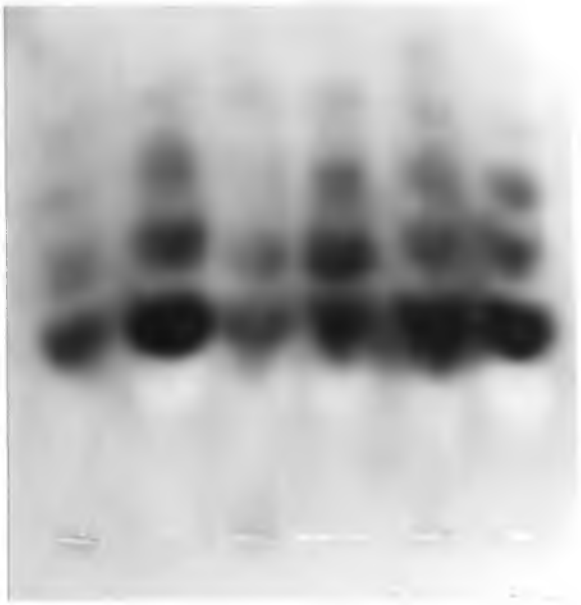


FIGURE 6. Phosphoglucomutase (PGM) isozyme patterns in human host livers (HL) and their corresponding hepatomas (H).

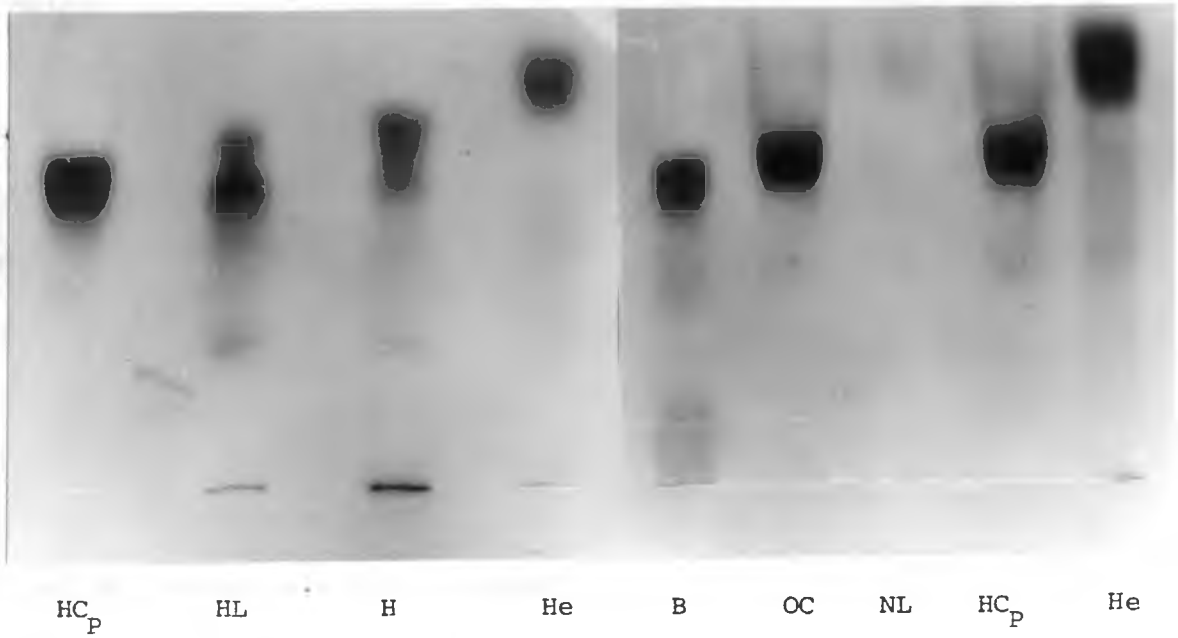
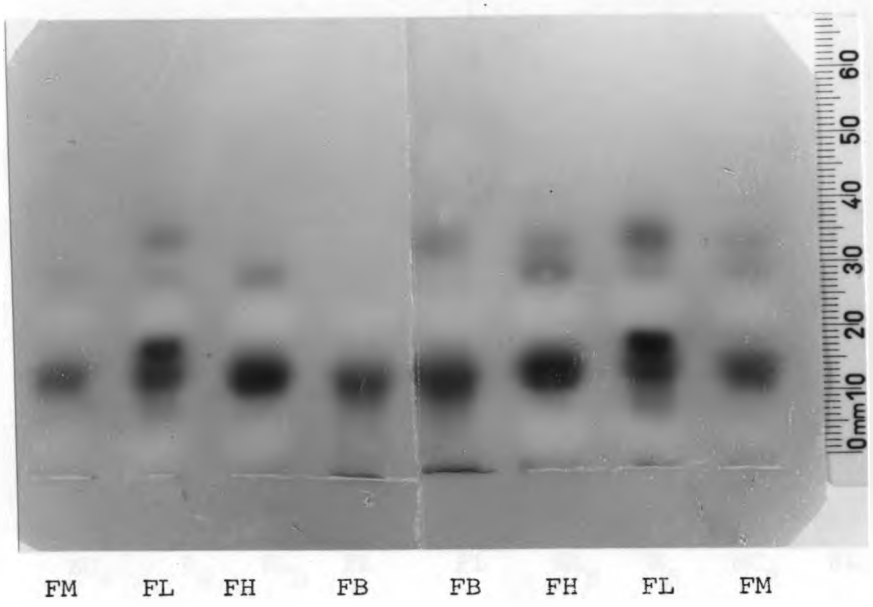


FIGURE 7. Glucose-6-phosphate dehydrogenase (G6PD) isozymes in human hepatoma cells (HC_P), surgical host liver (HL) and the corresponding hepatoma (H), HeLa cells (He), blood-G6PD B (B), oesophageal cancer cells (OC) and normal liver - G6PD A (NL).



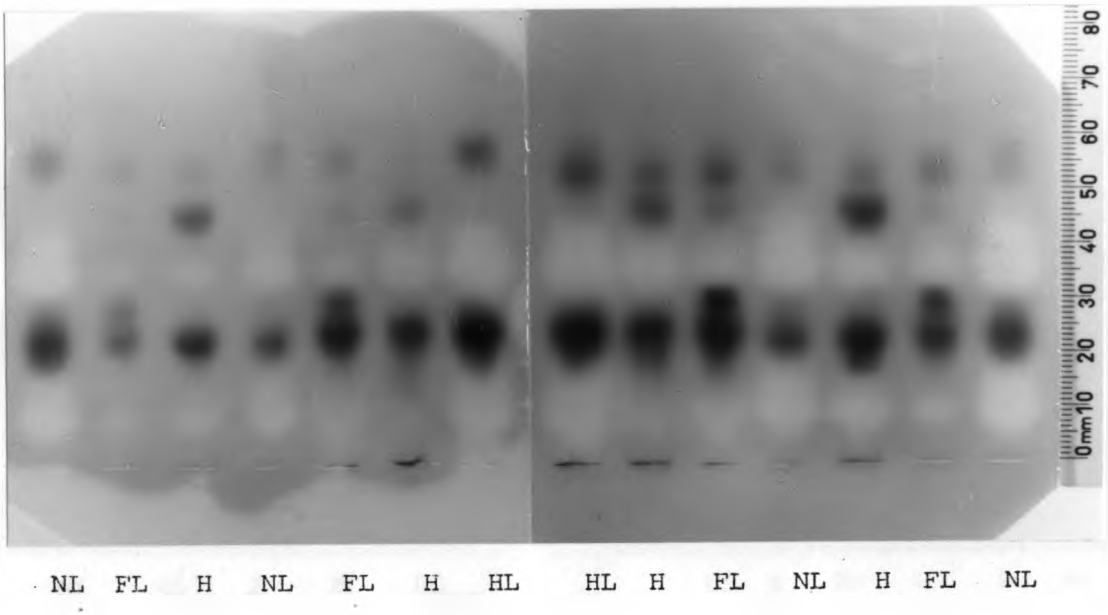


FIGURE 9. Hexokinase (HK) isozyme patterns in human foetal muscle (FM), liver (FL), heart (FH) and brain (FB), normal adult liver (NL), host liver (HL) and hepatoma (H). Electrophoresis was carried out at 1.2 mA/cm gel width (top photograph) or 1.7 mA/cm gel width (bottom photograph). The gels on the left of each photograph were stained with 0.5 mM glucose and the corresponding halves on the right with 0.1 M glucose.

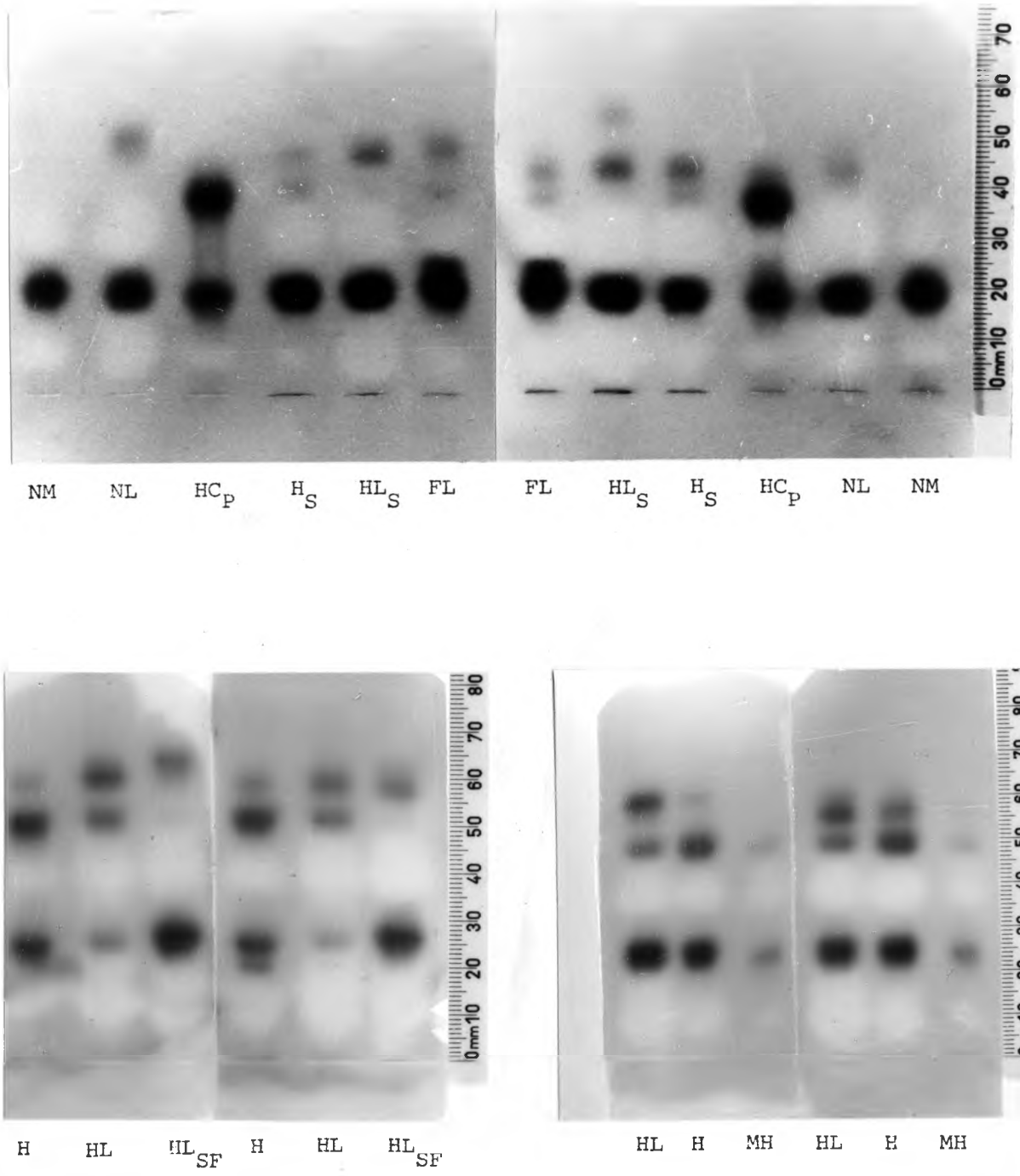


FIGURE 10. Hexokinase (HK) isozyme patterns in human normal muscle (NM), normal liver (NL), hepatoma cells (HC_P), host liver (HL), hepatoma (H) and foetal liver (FL). Host liver and hepatoma obtained during surgery are represented as HL_S and H_S, respectively. MH is muscle obtained from a hepatoma patient. Fresh tissues were used except for normal muscle and liver which had been frozen; HL_{SF} is the surgical host liver rerun after the tissue had been frozen. Electrophoresis was carried out at 1.5 (top photograph) or 1.6 - 1.7 (bottom photographs) mA/cm gel width. The gels on the left of each photograph were stained with 0.5 mM glucose and the corresponding halves on the right with 0.1 M glucose.

had the G6PD A isozyme. In hepatoma cells (A), however, G6PD was present as the A form, as in the original tumour from which they were derived. The mobility of G6PD A of HeLa cells increased slightly after the cell extract had been frozen for several months.

Hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH)

The HK, PK and LDH isozyme patterns in foetal heart, brain, muscle and liver, adult muscle, livers, hepatoma, hepatoma cells, oesophageal cancer, oesophageal cancer cells, normal dura, meningioma, mesothelioma, HeLa cells and fibroblast cells are summarised diagrammatically in Fig. 8.

In most cases, the patterns shown for each tissue have been compiled from studies carried out on material derived from a number of different individuals. Tissue-specific differences in foetal isozyme patterns were apparent for HK and PK but not for LDH. There did not appear to be any age-related differences in the isozyme patterns in livers and brains of six foetuses ranging from 10 - 28 weeks gestation. Heart was obtained from four foetuses (16 - 28 weeks) and muscle from two foetuses (12 and 20 weeks) and the patterns in these tissues also did not vary with age. There was hardly any variation in the patterns obtained for normal adult muscle and liver derived from three different individuals. HK and PK isozyme patterns were studied in eight host livers and their corresponding hepatomas as well as in several other hepatoma tissues. The patterns shown for HK depict the general trends which were apparent in all host livers and hepatomas but those shown for PK are typical of only a few cases since there were no consistent trends. There was only occasional variation in the LDH isozyme patterns obtained for six host livers and hepatomas and those shown are typical of the majority of cases. Oesophageal

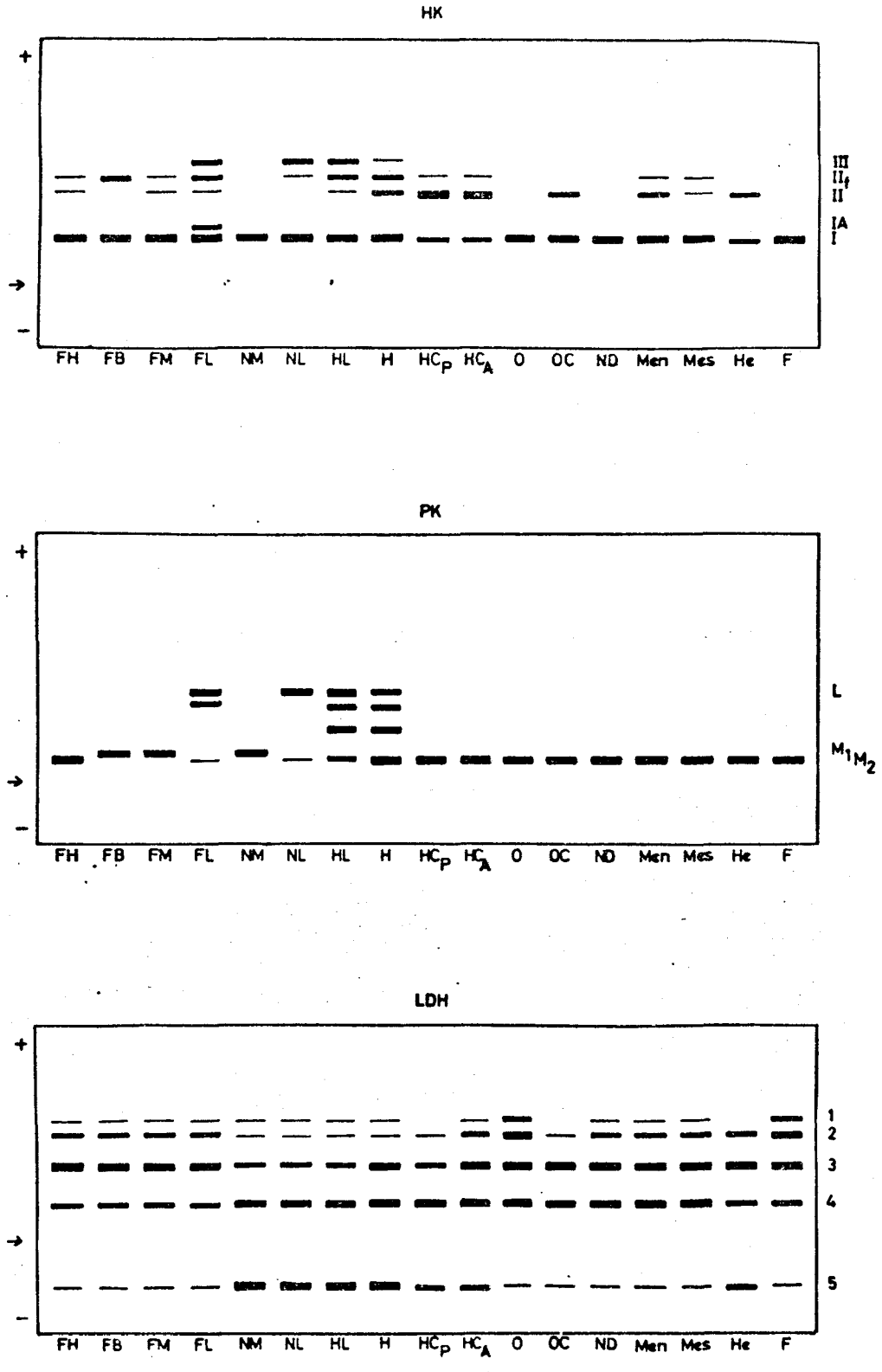


FIGURE 8. Diagrammatic representation of hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) isozyme patterns in human foetal heart (FH), foetal brain (FB), foetal muscle (FM), foetal liver (FL), normal muscle (NM), normal liver (NL), host liver (HL), hepatoma (H), hepatoma cells (HC_P and HC_A), oesophageal cancer (O), oesophageal cancer cells (OC), normal dura (ND), meningioma (Men), mesothelioma (Mes), HeLa cells (He) and fibroblast cells (F). The origin is indicated by an arrow.

cancer was obtained from two and meningioma from three patients and the respective patterns were the same for each patient. Normal dura and mesothelioma (derived from the visceral and parietal pleura) were each obtained from only one patient. The patterns in cell cultures were examined on several occasions and were found to be consistent.

Hexokinase

Examples of some of the HK isozyme patterns obtained are shown in Figs. 9, 10 and 11. When electrophoresis was carried out initially using a current of 1.1 - 1.2 mA/cm gel width, as in the previous studies, it was noticed that HK III of foetal liver and also of host liver and hepatoma appeared to stain more heavily in the presence of 0.1 M glucose than in the presence of 0.5 mM glucose, whereas in normal liver this isozyme was inhibited at the higher glucose concentration. In foetal muscle, heart and brain, HK III was not detected in gels stained with 0.5 mM glucose but was apparently present in gels stained with 0.1 M glucose (Fig. 9). Increasing the current to 1.5 - 1.8 mA/cm gel width improved the separation of the isozymes and it was then clear that HK III of foetal liver, host liver and hepatoma was in fact inhibited at the higher glucose concentration and that it was not HK III which was present in foetal muscle, heart and brain. A different isozyme, referred to as HK II_f, of mobility intermediate between HK II and HK III was present in the tissues. This isozyme stained heavily in the presence of 0.1 M glucose and only faintly, if at all, in the presence of 0.5 mM glucose. In subsequent experiments, electrophoresis was carried out using the higher current and HK II_f was also detected in other tissues and in cell cultures.

HK II_f was detected in foetal and adult livers whether or not

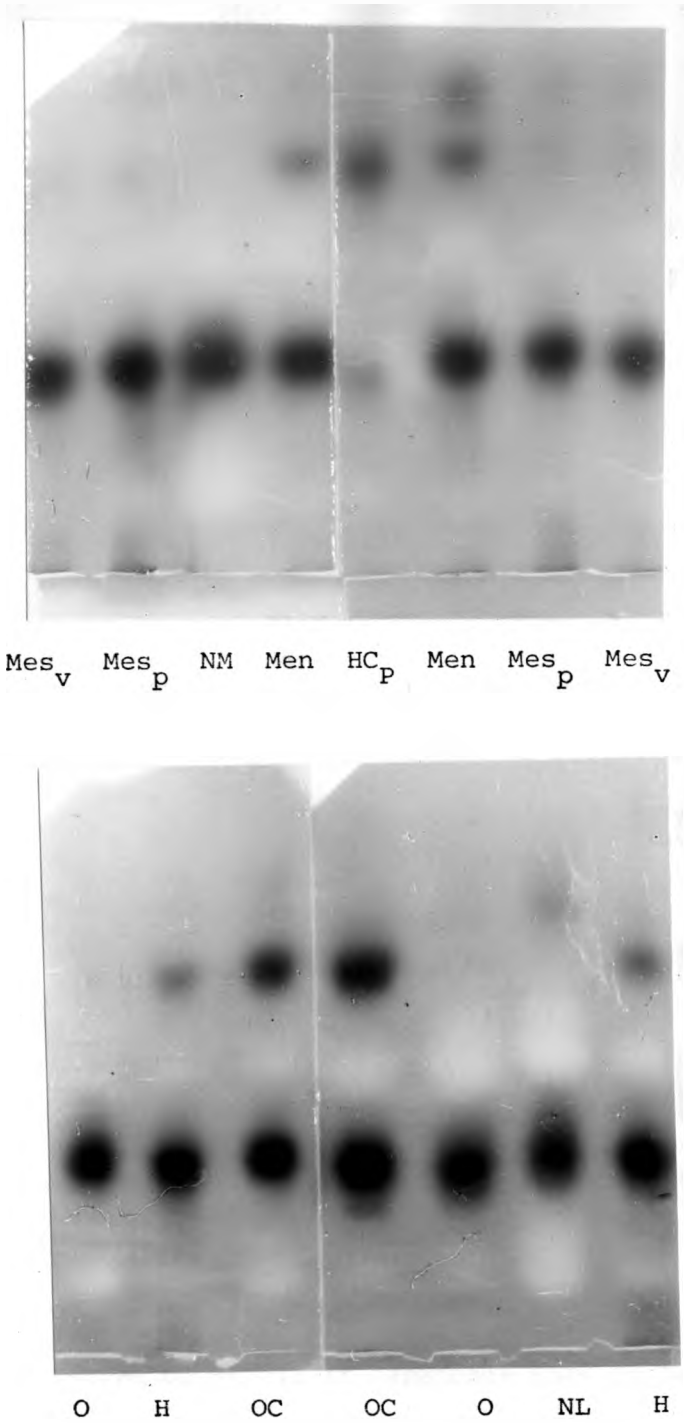


FIGURE 11. Hexokinase (HK) isozyme patterns in human mesothelioma, visceral pleura (Mes$_v$), mesothelioma, parietal pleura (Mes$_p$), normal muscle (NM), meningioma (Men), hepatoma cells (HC$_p$), oesophageal cancer (O), oesophageal cancer cells (OC), normal liver (NL) and hepatoma (H). Electrophoresis was carried out at 1.7 mA/cm gel width. The gels on the left on each photograph were stained with 0.5 mM glucose and those on the right with 0.1 M glucose.

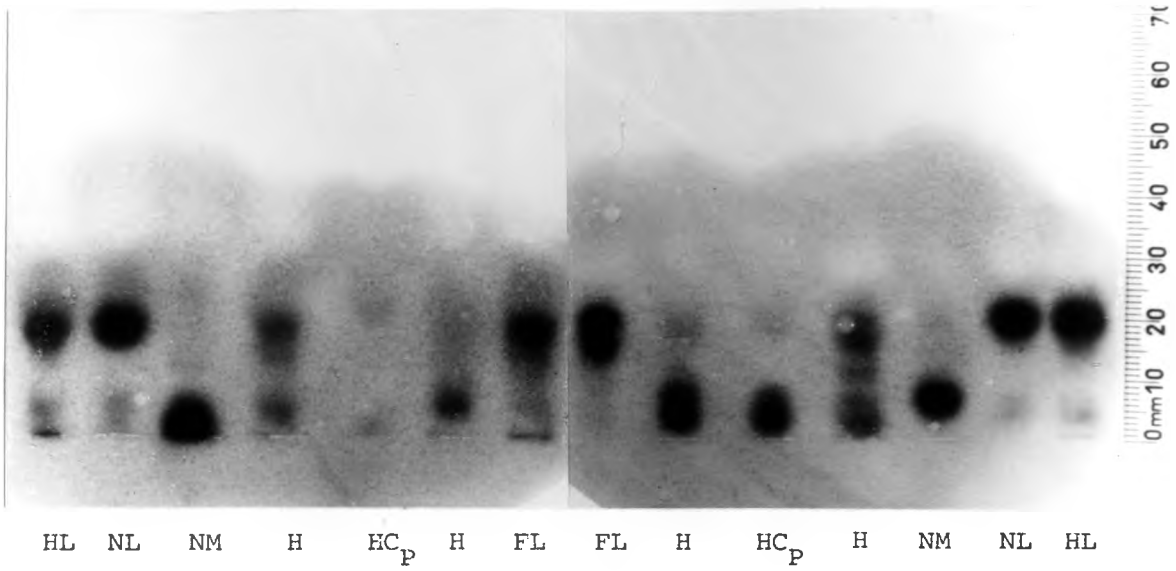


FIGURE 12. Effect of fructose 1,6-diphosphate (FDP) on human pyruvate kinase (PK) isozyme patterns. The gel on the left was run in the absence of FDP and the gel on the right had 0.1 mM FDP in the gel and electrode buffers. Electrophoresis was carried out at 1.6 mA/cm gel width. (HL - host liver, NL - normal liver, NM - normal muscle, H - hepatoma, HC$_P$-hepatoma cells, FL - foetal liver).

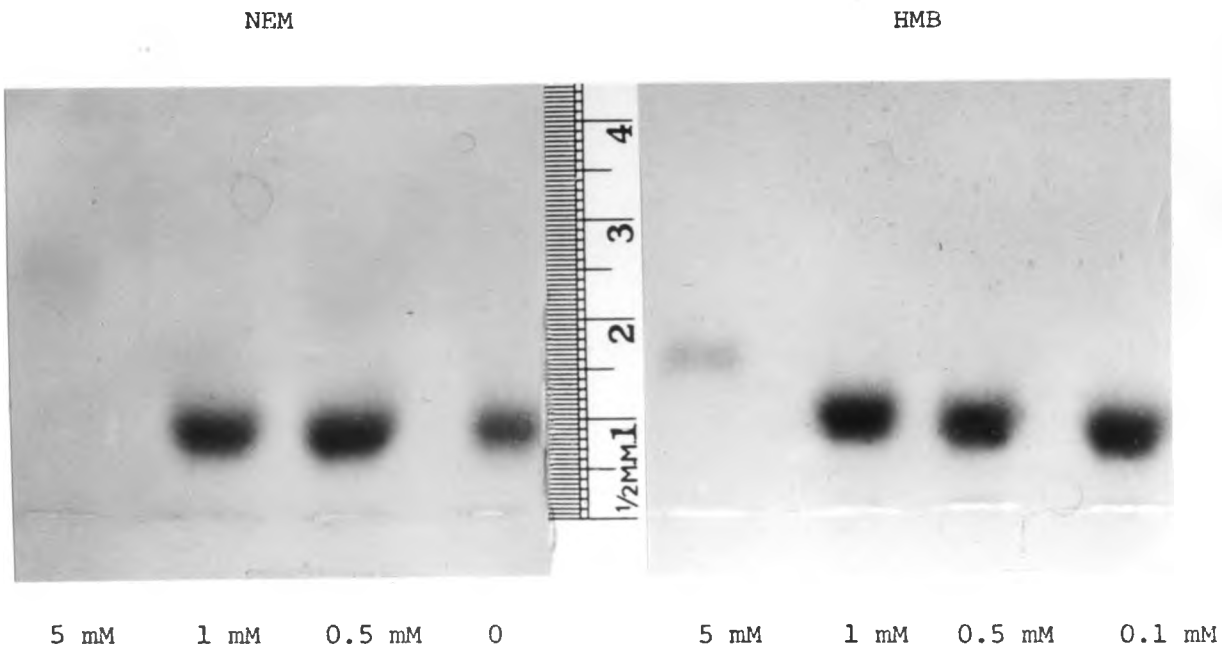


FIGURE 13. Effect of N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (HMB) on human muscle PK M$_1$. Before electrophoresis, muscle extracts were preincubated for 1 hour at 30° with NEM or HMB at the concentrations shown. Electrophoresis was carried out at 1.6 mA/cm gel width and DTT was omitted from the gel and electrode buffers.

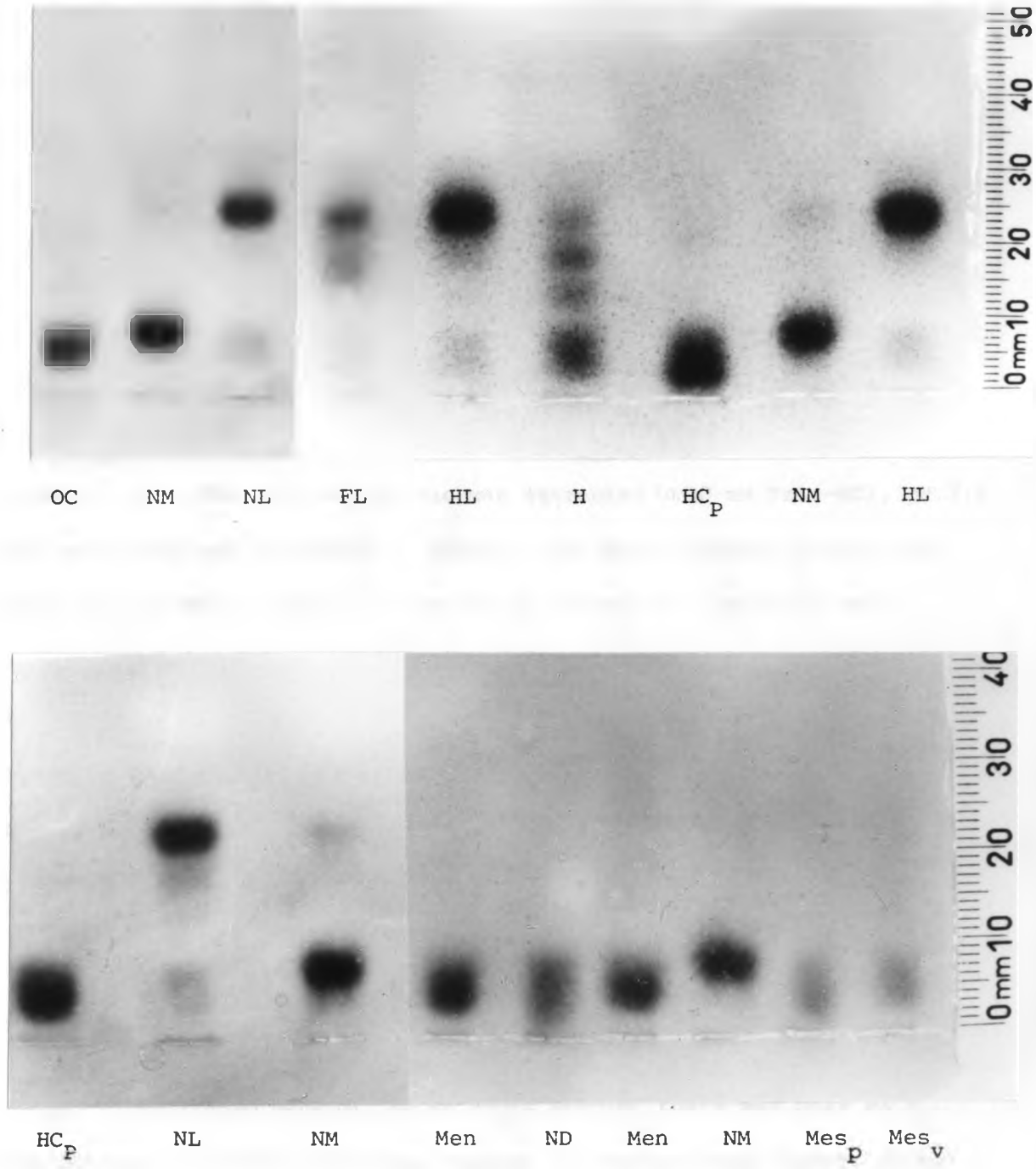


FIGURE 15. Pyruvate kinase (PK) isozyme patterns in human oesophageal cancer cells (OC), normal muscle (NM), normal liver (NL), foetal liver (FL), host liver (HL), hepatoma (H), hepatoma cells (HC_p), normal dura (ND), meningioma (Men), mesothelioma, parietal pleura (Mes_p), and mesothelioma, visceral pleura (Mes_v). Electrophoresis was carried out at 1.6 - 1.8 mA/cm gel width and 0.1 mM FDP was included in the gel and electrode buffers.

EDTA was included in the gel and electrode buffers, although in the absence of EDTA all the isozymes stained much more faintly.

It was also apparent, when electrophoresis was carried out using the higher current, that whilst the other isozymes were unaffected, the mobility of HK III was increased in tissues which had been stored frozen at -20°. This is shown in Fig. 10 for normal and surgical host liver.

Normal adult muscle had only the HK I isozyme and normal liver had HK I, which was the predominant form, HK II_f (a trace) and HK III.

The predominant isozyme in foetal tissues was HK I. In addition to HK I, traces of HK II and HK II_f were detected in foetal muscle and the isozymes, HK I, HK IA, HK II (a trace), HK II_f and HK III were present in foetal liver. The proportion of HK II_f in foetal liver was much greater than in normal liver. The pattern in foetal heart was similar to that of foetal muscle and foetal brain had HK I and HK II_f.

The patterns in all host livers and hepatomas differed from the normal liver pattern in that HK II was present and the proportion of HK II_f was greater. Although there was some variability in the actual amounts of the isozymes, it was found that in all cases there was a greater proportion of HK II relative to HK III in hepatomas than in host livers. As in normal liver, HK I was usually the major isozyme in host livers and hepatomas but in one case, in both host liver and hepatoma, HK II was the major form and in another HK II was the major form in the hepatoma but not in the corresponding host liver. The patterns in the host liver and hepatoma obtained during surgery resembled those of autopsy tissues except that HK IV was detected in the fresh host liver (Fig. 10). This was the only time that this isozyme was found in any human material and it was not detected after the tissue had been frozen. Muscle derived from

hepatoma patients was examined on four occasions and HK I was the only isozyme, except in one case when there was also a trace of HK II (Fig. 10).

The patterns in both the hepatoma cell lines differed from hepatoma patterns in that HK II was the predominant isozyme, the proportion of HK II_f was decreased and HK III was absent. The original tumour from which hepatoma cells (A) were derived had HK I, HK II, HK II_f and HK III with HK I the predominant isozyme. There was only HK I in oesophageal cancer tissue, whereas oesophageal cancer cells had HK II in addition to HK I.

In normal dura only HK I was detected whereas meningioma had HK II and HK II_f in addition to HK I. The pattern in mesothelioma tissue, derived from either the visceral or parietal pleura, was similar to that in meningioma.

The HK isozymes I and II were present in HeLa cells and in fibroblasts growing in tissue culture only HK I was detected.

Pyruvate kinase

Some of the actual PK isozyme patterns of the various tissues and cell cultures are shown in Figs. 12 - 15. Storage of tissues or cell extracts at -20° did not appear to affect the patterns.

As in rat, the PK isozymes M_1 and M_2 were not separated when electrophoresis was carried out using a current of 1.1 - 1.2 mA/cm gel width, but could be distinguished when the current was increased to 1.6 - 1.8 mA/cm gel width. The separation of the M_1 and M_2 isozymes was not improved further by increasing the pH of the gel and electrode buffers to 9.3.

The effect of including 0.1 mM fructose-1,6-diphosphate (FDP)

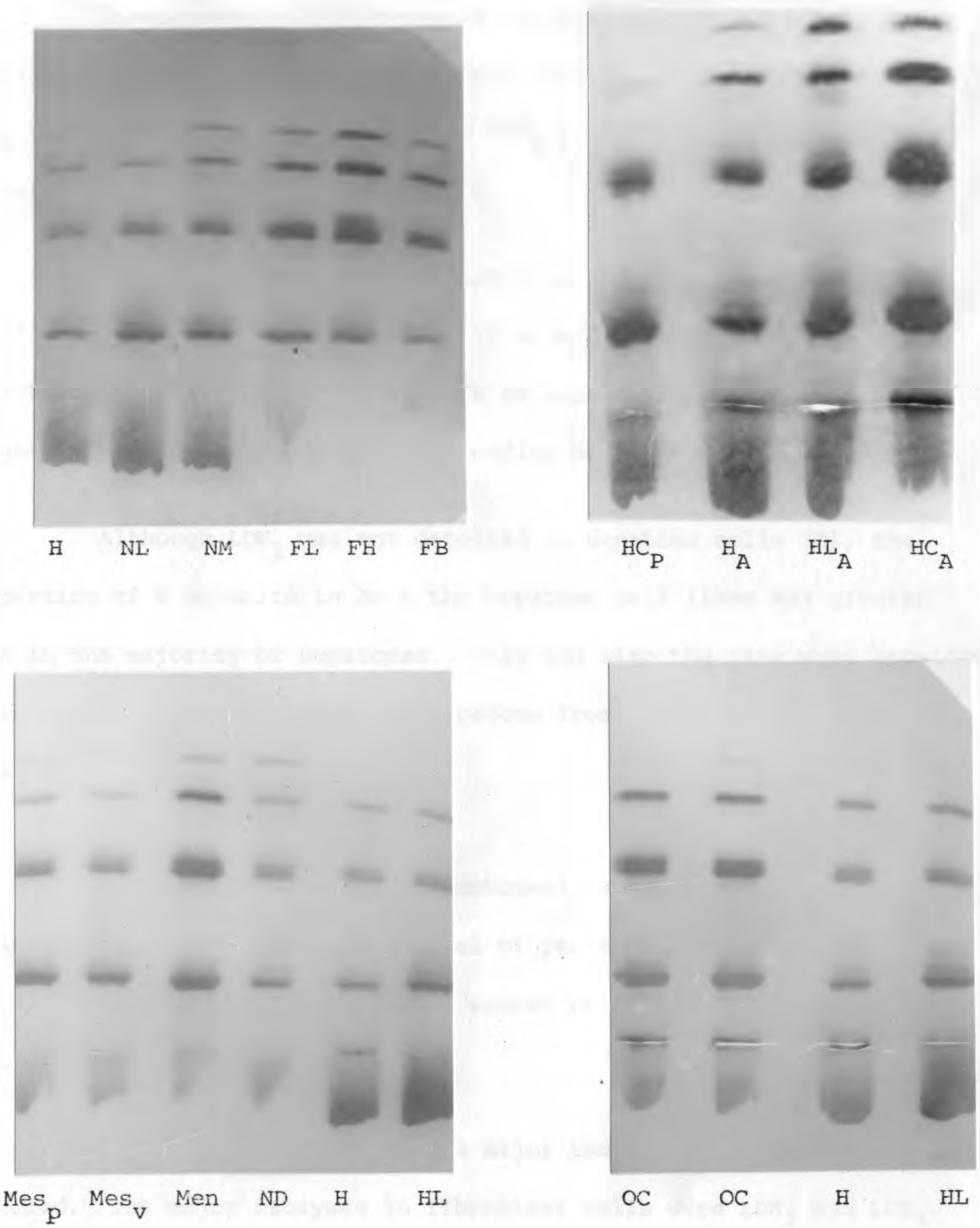
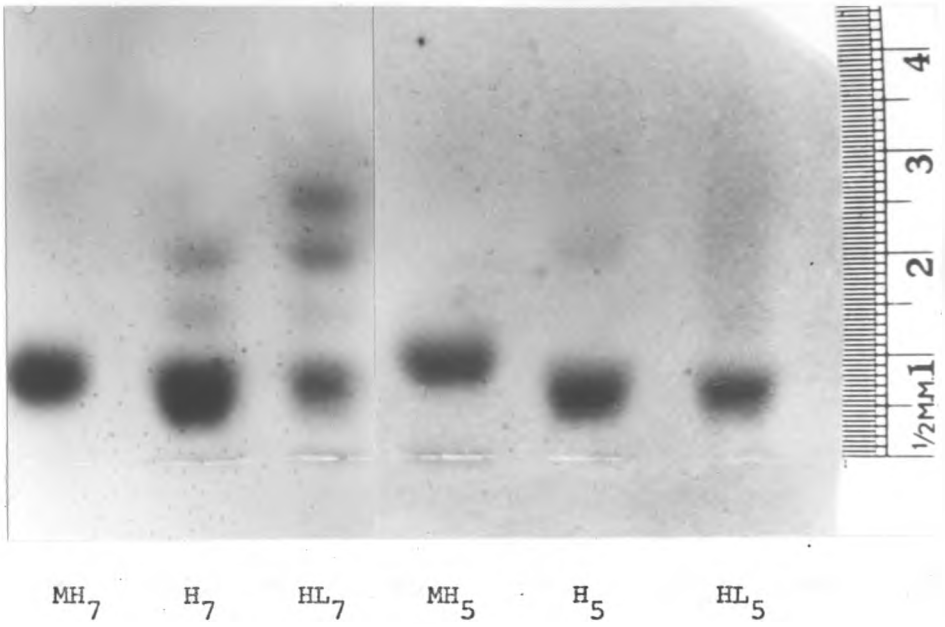


FIGURE 16. Lactate dehydrogenase (LDH) isozyme patterns in human hepatoma (H), normal liver (NL), host liver (HL), normal muscle (NM), foetal liver (FL), foetal heart (FH), foetal brain (FB), hepatoma cells (HC_P and HC_A), hepatoma from which hepatoma cells (A) were derived and its corresponding host liver (H_A and HL_A, respectively), mesothelioma, parietal pleura (Mes_p), mesothelioma, visceral pleura (Mes_v), meningioma (Men), normal dura (ND) and oesophageal cancer cells (OC).



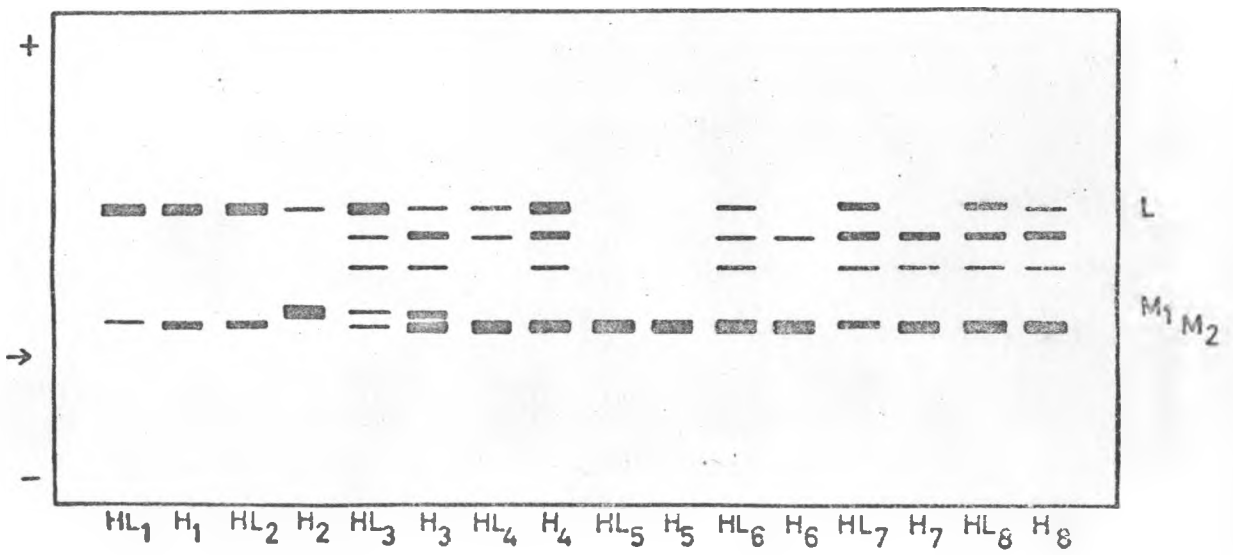


FIGURE 14. Pyruvate kinase (PK) isozyme patterns in human host livers (HL) and their corresponding hepatomas (H) and also in muscle derived from hepatoma patients (MH). Electrophoresis was carried out at 1.6 - 1.8 mA/cm gel width and 0.1 mM FDP was included in the gel and electrode buffers.

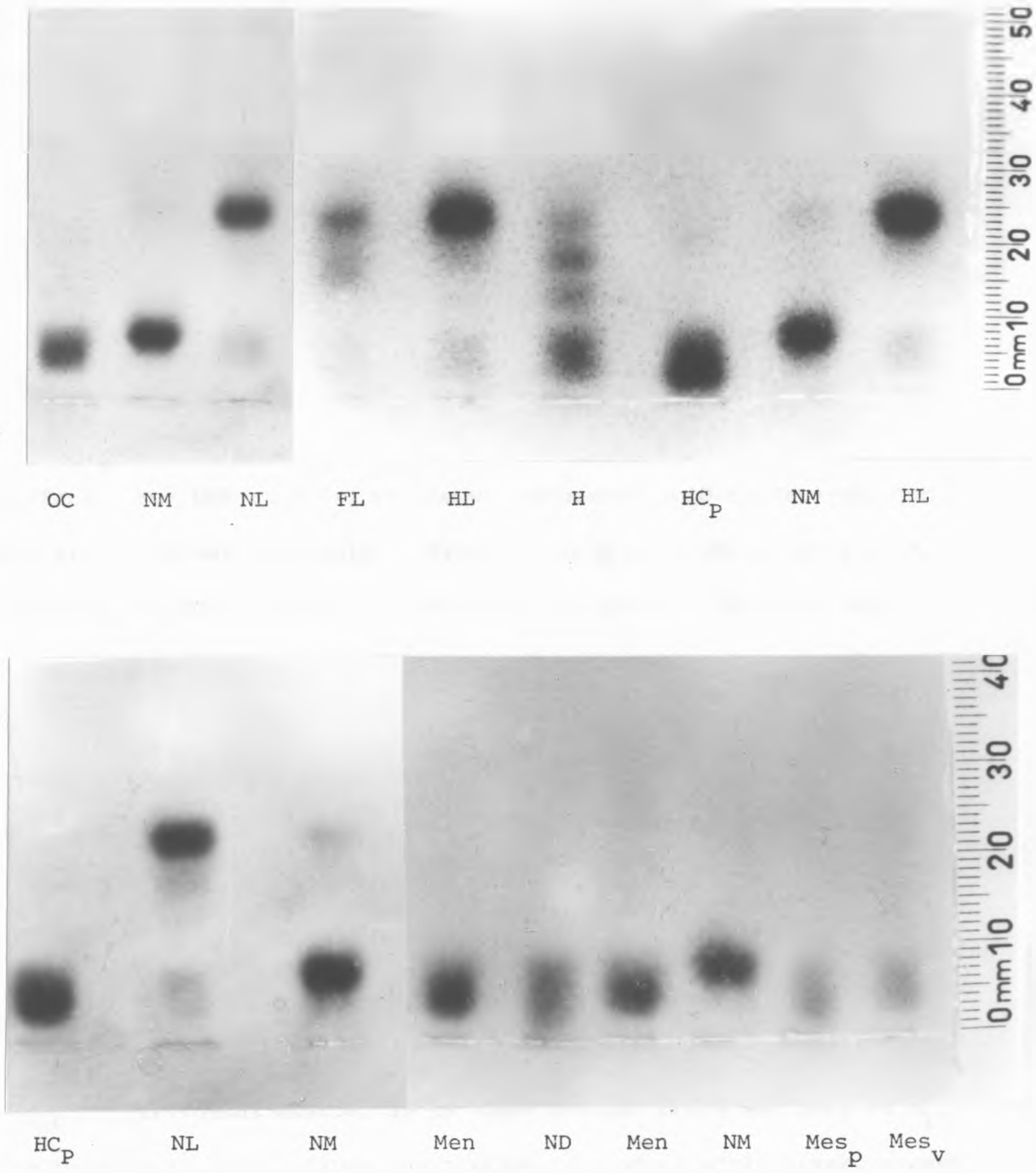


FIGURE 15. Pyruvate kinase (PK) isozyme patterns in human oesophageal cancer cells (OC), normal muscle (NM), normal liver (NL), foetal liver (FL), host liver (HL), hepatoma (H), hepatoma cells (HC_p), normal dura (ND), meningioma (Men), mesothelioma, parietal pleura (Mes_p), and mesothelioma, visceral pleura (Mes_v). Electrophoresis was carried out at 1.6 - 1.8 mA/cm gel width and 0.1 mM FDP was included in the gel and electrode buffers.

in the gel and electrode buffers is shown in Fig. 12. In the absence of FDP, PK M_1 remained at the origin and PK M_2 was slightly faster, whereas in the presence of FDP, PK M_1 migrated away from the origin and was slightly faster than the M_2 isozyme. The isozymes stained more clearly and the separation was better in the presence of FDP.

The effects of N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (HMB) on PK M_1 of muscle are shown in Fig. 13. For these experiments DTT was omitted from the extraction buffer and from the gel and electrode buffers. For NEM studies, tissue was extracted in 10 mM Tris-HCl, pH 7.4 but since HMB was insoluble in Tris, 50 mM glycylglycine, pH 8.0 was used for the HMB studies. There was no change when extracts were preincubated for 1 hour at 30° with 0.5 or 1 mM NEM, but with 5 or 10 mM NEM the isozyme was completely inhibited. However, after extracts had been preincubated for 1 hour at 30° with 0.1, 0.5, 1 and 5 mM HMB, the mobility of PK M_1 increased with increasing HMB concentration, although at 5 mM HMB there was considerable inhibition.

In normal muscle, only PK M_1 was detected. The major isozyme of normal liver was PK L and there was a small amount of PK M_2.

In foetal muscle, as in adult muscle, there was only PK M_1. The pattern in foetal liver was similar to that of adult liver, except that an additional band of mobility slightly less than that of PK L, was detected in several cases. The presence of this isozyme did not correlate with foetal age. The only isozymes detected in foetal heart and brain were PK M_2 and PK M_1, respectively.

The patterns in host livers and hepatomas were extremely variable, some examples are shown in Figs. 12, 14 and 15, and a diagrammatic representation is given in Fig. 14. There were no consistent differences

between host liver and hepatoma and the patterns of both were usually different from the normal liver pattern. In one case PK L was the major form in both host liver and hepatoma and in another both tissues had only the M_2 isozyme. One of the hepatomas appeared to have the M_1 isozyme and another appeared to have a mixture of the M_1 and M_2 forms. Several host livers and hepatomas showed two additional strongly-staining bands between PK L and PK M_2 and in one case a third additional band, similar to PK M_1, was detected.

Muscle from four hepatoma patients was examined and in three cases there was only the M_1 isozyme but in the fourth there were four bands as in the corresponding hepatoma.

Both hepatoma cell lines had only PK M_2, whereas the original tumour tissue from which hepatoma cells (A) were derived appeared to have the M_1 isozyme.

In oesophageal cancer and oesophageal cancer cells, there was only the M_2 isozyme and this was also the case for normal dura, meningioma, mesothelioma (visceral or parietal pleura), HeLa cells and fibroblasts.

Lactate dehydrogenase

Examples of LDH isozyme patterns are shown in Fig. 16. The patterns did not appear to be affected by storing tissues or cell extracts at -20°. Human tissues had all five LDH isozymes but in some of the cell cultures LDH_1 was not detected.

The patterns in normal muscle and liver were similar, LDH_5 and LDH_4 had the greatest intensities and the intensities of LDH_3, LDH_2 and LDH_1 decreased with increasing mobility.

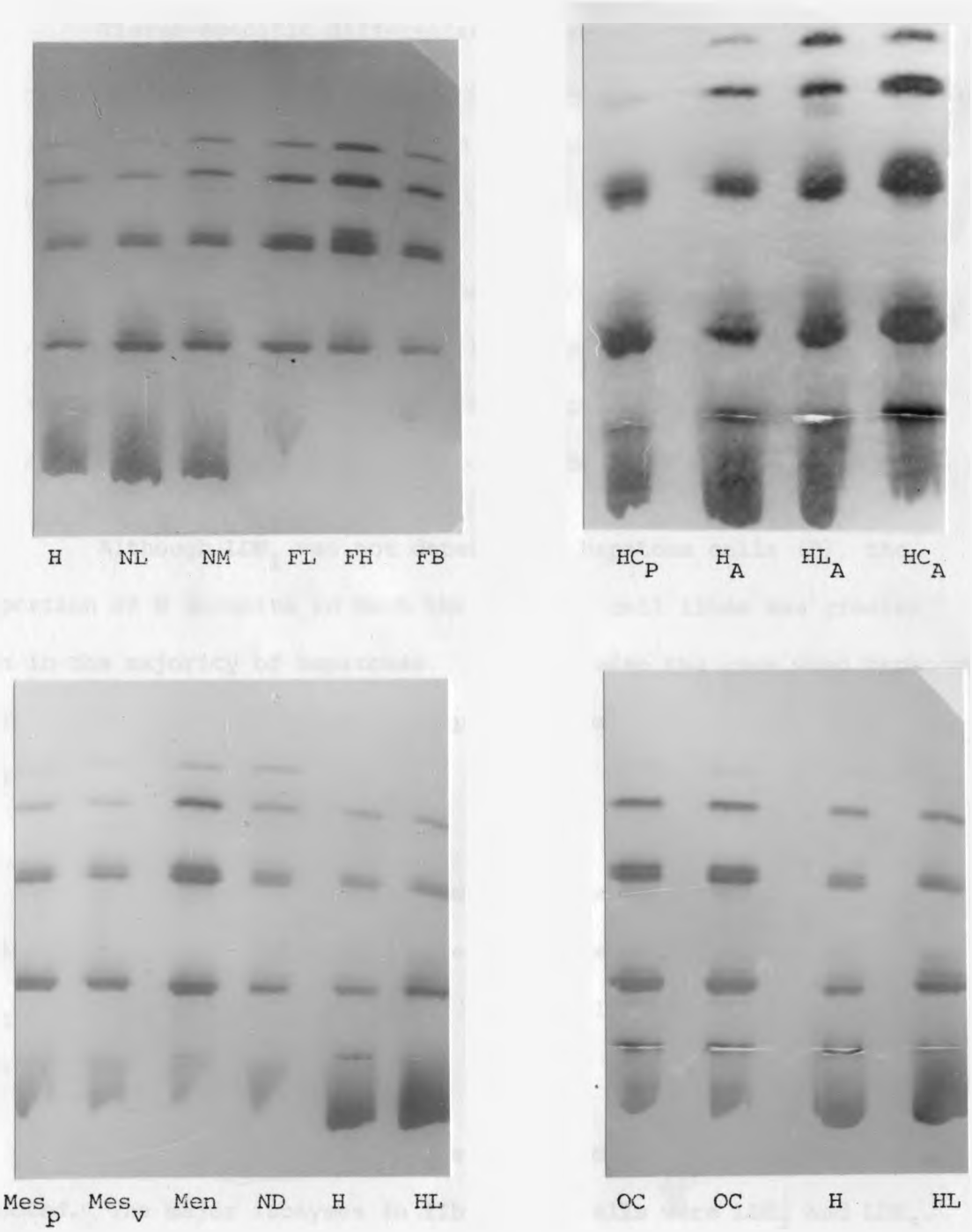


FIGURE 16. Lactate dehydrogenase (LDH) isozyme patterns in human hepatoma (H), normal liver (NL), host liver (HL), normal muscle (NM), foetal liver (FL), foetal heart (FH), foetal brain (FB), hepatoma cells (HC_P and HC_A), hepatoma from which hepatoma cells (A) were derived and its corresponding host liver (H_A and HL_A, respectively), mesothelioma, parietal pleura (Mes_p), mesothelioma, visceral pleura (Mes_v), meningioma (Men), normal dura (ND) and oesophageal cancer cells (OC).

Tissue-specific differences between the patterns in foetal heart, brain, muscle and liver were not apparent. In all four tissues, LDH_3 was the major isozyme, LDH_2 and LDH_4 were present and there were traces of LDH_1 and LDH_5.

In nearly all cases, the patterns in host livers and hepatomas resembled those of normal liver and muscle. Occasionally, however, the proportion of H subunits in hepatoma appeared to be slightly greater than in the corresponding host liver or normal liver.

Although LDH_1 was not detected in hepatoma cells (P), the proportion of H subunits in both the hepatoma cell lines was greater than in the majority of hepatomas. This was also the case when hepatoma cells (A) were compared with the hepatoma from which they were derived, as is shown in Fig. 16.

The major isozymes in oesophageal cancer, normal dura, meningioma and mesothelioma (visceral or parietal pleura) were LDH_3 and LDH_4 and the pattern in oesophageal cancer cells was very similar to that of oesophageal cancer tissue.

HeLa cells had LDH_3 as the major isozyme and LDH_1 was not detected. The major isozymes in fibroblast cells were LDH_2 and LDH_3.

ISOELECTRIC FOCUSING

Protein patterns

Clear protein patterns were obtained using Ampholines in the pH ranges 3 - 10 or 5 - 8.

After isoelectric focusing, human normal and host livers showed seven sharply defined haemoglobin bands in the pH region 6.9 - 7.6, whereas only one or two bands were apparent in hepatoma (Fig. 17A). When the gels were stained for protein, numerous bands were visible and the hepatoma pattern differed from that of host liver as shown in Fig. 17A. In hepatoma, there were fewer bands in the pH region 6.5 - 7.5 and more in the pH region 4.5 - 5.5 than in liver.

Isozyme patterns

For isozyme studies, the pH range 3 - 10 was found to be more satisfactory than the range 5 - 8, since the bands were much more clearly defined using the wider range.

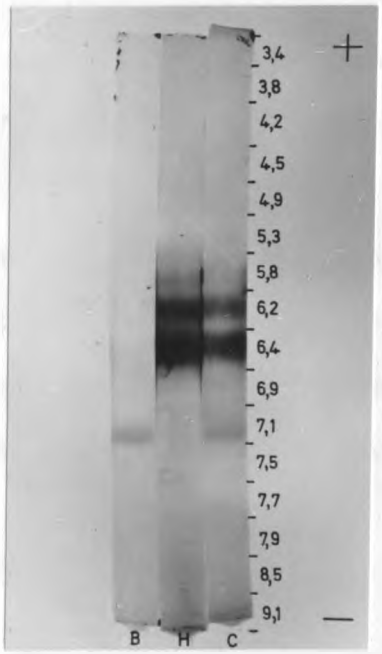
Staining the gels for enzyme activity presented two problems. Firstly, the bands could not be fixed at the end of the run, (e.g. with trichloroacetic acid) as this destroyed enzyme activity, and therefore diffusion took place during staining so that the bands were much less clearly defined than when the protein stain was used. Secondly, Ampholines are chelating agents and chelate the cations which are included in some of the stains (e.g. for HK), to enhance enzyme activity. This resulted in longer staining times and increased diffusion of the bands. Removal of Ampholines by dialysis, prior to staining also resulted in increased diffusion. Increasing the divalent cation concentration when staining for HK was not found to reduce the staining time significantly. This technique was therefore found to be most satisfactory for enzymes with relatively high activity which do not require metal ions for activation (e.g. PGM and LDH), so that staining times were as short as possible.

A



H_H C_H H_P C_P H_L C_L

B



C

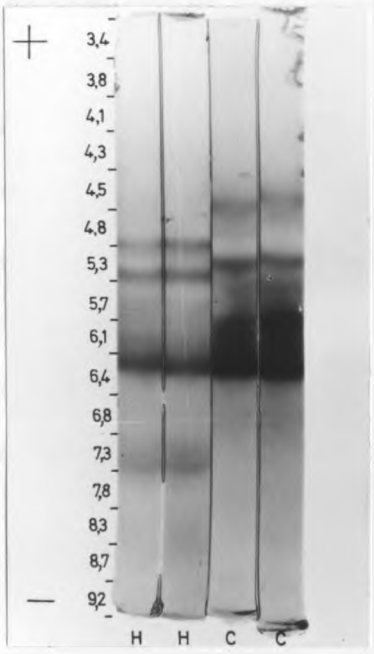


FIGURE 17. Disc gel isoelectric focusing in the pH range 3-10.

A. Haemoglobin bands in human liver (H_H) and hepatoma (C_H). Protein patterns in human host liver (H_P) and hepatoma (C_P). Lactate dehydrogenase patterns in fresh human host liver (H_L) and hepatoma (C_L).

B. Phosphoglucomutase (PGM) patterns in human host liver (H) and hepatoma (C). (B - blank).

C. Hexokinase (HK) patterns in rat host liver (H) and hepatoma (C).

Phosphoglucomutase

Rat normal and host livers and hepatomas showed three or four bands (often with sub-banding) in the region pH 5.0 - 6.6. Although on occasions there were slight differences between the patterns in different tissues, there were no consistent changes. Patterns in human host livers and hepatomas also consisted of a series of bands in the region pH 5.0 - 6.6, though the positions varied slightly according to the phenotype (an example is shown in Fig. 17B). There were no consistent differences between human host liver and hepatoma patterns.

Hexokinase

An example of the HK patterns in rat host liver and hepatoma is shown in Fig. 17C. Rat normal and host liver had either two or three bands of HK activity in the pH range 5.0 to 6.4, whereas in hepatoma there was also a weak more anodic band. In livers and hepatomas, the most intense band was the cathodic one (pH 6.4). The intensities of the more cathodic bands were greater in hepatoma than in liver. On staining with 0.1 M glucose, no additional forms were detected in any of the tissue extracts.

Lactate dehydrogenase

When gels were stained for LDH activity, a major cathodic band (pH 8.3 - 9) and a few fainter anodic bands were apparent in rat host liver, whereas in hepatoma there was also a cathodic band which was shifted to a higher pH and a number of bands ($\sim$ 8) in the pH region 4.8 - 6.8. In normal human liver which had been frozen there were 12 bands in the region pH 4.7 - 6.5 as well as a major cathodic band. Human hepatoma, which had also been frozen had fewer anodic bands and there were slight differences in the positions of these compared with normal liver. However,

in fresh human host liver the major cathodic band was split into three and the anodic bands were absent; the corresponding hepatoma had, in addition to the major band, one stronger and three weak anodic bands (Fig. 17A).

DISCUSSION

Comparative studies showed that the electrophoretic mobilities of the isozymes of phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) varied from species to species. There were marked differences between rat, human and bovine isozyme mobilities but vervet monkey and baboon isozymes ran similarly to the corresponding human forms. Species differences in mobilities of PGM, G6PD, HK, PK and LDH isozymes have been demonstrated by others (Markert and Møller 1959; Vesell and Brody, 1964; Lauris and Cahill, 1966; Grossbard *et al.*, 1966; Montes de Oca *et al.*, 1969; Dawson and Jaeger, 1970; Osterman and Fritz, 1973; Whittell *et al.*, 1973; Dawson and Greene, 1975). The differences between the mobilities of the G6PD and LDH isozymes in the species studied corresponded with those reported by Montes de Oca *et al.* (1969). Grossbard *et al.* (1966) showed that, as in this study, vervet monkey HK isozymes were slightly faster than the rat forms and they also noted differences between the mobilities of the HK isozymes of vervet and rhesus monkey. The human PK isozymes migrated more slowly than the corresponding rat forms, which is in agreement with the findings of Osterman and Fritz (1973).

Cross-contamination is known to occur frequently in tissue culture and studies of isozyme mobilities are of importance in confirming the species origin of cell lines (Gartler, 1967; Montes de Oca *et al.*, 1969; Gartler and Farber, 1973; Shannon and Macy, 1973). This was well illustrated in one instance, when comparative electrophoretic studies showed that contamination had occurred during culture and what were presumed to be human hepatoma cells were shown actually to be bovine kidney (MDBK) cells. The mobilities of the G6PD, HK, PK and LDH isozymes in other rat and human cell lines used in this study corresponded with those in tissues of the

species of origin. Moreover, rat and human cells were easily distinguished from one another and from cells of bovine origin. It was, however, only possible to distinguish between human and monkey cells if the human cells had the G6PD B isozyme. Similarly, Montes de Oca *et al.* (1969) showed that rat, human and bovine cells as well as cells derived from a number of other species could easily be distinguished by means of their G6PD and LDH isozyme mobilities, but that the mobilities of the human and monkey LDH isozymes were similar and the monkey G6PD isozyme ran similarly to human G6PD A.

It was also not possible to distinguish between human cells which had the G6PD A isozyme and HeLa cells, which have been shown to be frequent tissue culture contaminants (Gartler, 1967, 1968). There were minor differences between the HK and LDH isozyme distributions in HeLa cells and other human cells but these were not adequate for a positive distinction to be made.

During LDH hybridisation studies isozymes which had different mobilities from those of the original tissues or cells were formed, indicating that, although the features required for the association of subunits into active tetramers were retained in the different species, there were differences in the nature of the subunits. Extensive LDH hybridisation studies have been carried out and inter-species hybrids have been obtained for a wide variety of other species (Markert, 1963; Chilson *et al.*, 1965; Fondy and Kaplan, 1965; Vesell, 1965; Salthe *et al.*, 1965).

Hybridisation of pig heart LDH_1 with human liver LDH_5 gave rise to five isozymes with an almost binomial distribution, as would be expected from the random recombination of two different types of subunit to form a tetrameric structure. Hybridisation of human LDH_5 with the LDH isozymes of

MDBK cells gave rise to ten or eleven bands and in fact fifteen bands would be expected on recombination of three non-identical, structurally and functionally equivalent subunits to form a tetrameric structure (Shaw, 1964). The isozymes missing appeared to be those involving combination of MDBK H subunits with each other or with only MDBK M subunits, and this would indicate preferential combination of the MDBK subunits with the M subunits of human liver. However, when pig heart LDH_1 was hybridised with the isozymes of MDBK cells ten or eleven bands were again produced but, since the bands in the more anodic region were close together, it was not clear which hybrids were missing, although those involving combination of MDBK H subunits with each other or with MDBK M subunits were present, perhaps suggesting preferential recombination of MDBK subunits with each other rather than with pig H subunits.

Studies of isozyme patterns in rat tissues showed that in the fairly fast-growing 3'-MeDAB-induced hepatoma, PGM, G6PD and LDH patterns were unaltered whereas liver-specific HK and PK isozymes were replaced by other forms. Changes in isozyme patterns in rat hepatomas are well documented and are described in reviews by Criss (1971) and Schapira (1973).

The PGM patterns in rat tissues consisted of a series of multiple forms and it seems likely, from studies carried out on rabbit, that such forms probably exist *in vivo* and are caused by progressive oxidation of free sulphydryl groups (Greene and Dawson, 1973; Dawson and Greene, 1975). Weber *et al.* (1964) had reported that PGM had a higher Michaelis constant for glucose-1-phosphate in a fast-growing hepatoma than in normal liver, whereas the value of this constant for a slower-growing hepatoma was similar to that obtained for normal liver. PGM from the fairly fast-growing hepatoma used in this work, however, had a similar Michaelis constant for glucose-1-phosphate to that obtained for normal liver. Since normal and host liver

and hepatoma showed similar PGM patterns on electrophoresis and the Michaelis constants for glucose-1-phosphate were similar in these tissues, it would seem that the reduced activity in the hepatoma (Balinsky *et al.*, 1972) results from a quantitative reduction in enzyme protein synthesis and not from an alteration in the enzyme protein itself.

The major G6PD isozyme of rat muscle had a slower electrophoretic mobility than the major liver isozyme which was frequently split into two sub-bands. The sub-bands of the liver isozyme presumably correspond to types I and II described by Taketa and Watanabe (1971) using polyacrylamide gel electrophoresis. In this work it was found that liver and hepatoma G6PD patterns were similar, but Taketa and Watanabe (1971) reported that, in addition to types I and II, a faster moving sub-band, type III, was present in hepatoma AH-30.

The HK isozyme patterns obtained for rat muscle and liver were similar to those reported by others (Katzen *et al.*, 1965; Katzen and Schimke, 1965; Grossbard *et al.*, 1966).

The increased activity of HK in the rat hepatoma, previously reported by others in this laboratory (Balinsky *et al.*, 1972), was found to be associated with alteration in isozyme pattern. In the hepatoma, the regulatory isozyme HK IV was not detected and the proportion of HK III decreased while that of HK II increased as compared with normal or host liver. Disappearance of HK IV was observed in fast-growing, poorly-differentiated hepatomas but in slow-growing, well-differentiated tumours this isozyme was retained (Sato *et al.*, 1969; Shatton *et al.*, 1969; Weinhouse *et al.*, 1972a; Sato and Sugimura, 1973). Sato and Sugimura (1973) detected a faster-moving HK IV isozyme in some hepatomas. Increases in the proportion of HK II in hepatomas of varying growth rates were observed by

Sato *et al.* (1969) but not by Shatton *et al.* (1969), Weinhouse *et al.* (1972a) or Sato and Sugimura (1973). The loss of the regulatory isozyme HK IV may indicate a loss of dietary and hormonal control in the tumours. On the basis of studies of the properties of rat liver HK isozymes (Grossbard and Schimke, 1966; González *et al.*, 1967), increases in the proportion of HK II in hepatomas could result in reduced inhibition by glucose and ADP and increased inhibition by glucose-6-phosphate.

The three PK isozymes L, M_1 and M_2 were separated in rat tissues and, as previously observed (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Whittell *et al.*, 1973; Osterman and Fritz, 1973), muscle had only PK M_1 and liver had PK L (the predominant form) and PK M_2. In liver, Imamura and Tanaka (1972), Whittell *et al.* (1973) and Osterman and Fritz (1973) have occasionally observed additional bands, possibly hybrids, between PK L and PK M_2.

The increased activity of PK in the rat hepatoma (Balinsky *et al.*, 1972) was found to be associated with almost total replacement of the L isozyme by the M_2 form. This is consistent with previous findings for fairly fast-growing hepatomas, but a substantial proportion of PK L was retained in slow-growing tumours (Schapira and Gregori, 1971; Imamura and Tanaka, 1972; Walker and Potter, 1972; Weinhouse *et al.*, 1972a; Farina *et al.*, 1974; Yanagi *et al.*, 1974; Schapira *et al.*, 1975). A cancer-specific isozyme has been observed in some hepatomas (Criss, 1969). The loss of the L form of PK in hepatomas is presumably associated with loss of certain regulatory features. The level of PK L is subject to nutritional and hormonal control whereas that of PK M_2 is not, and although both L and M_2 forms are allosterically regulated by FDP and ATP, the effects are less marked for the M_2 isozyme (Imamura *et al.*, 1972).

The predominant LDH isozyme in rat muscle and liver was LDH_5, as previously reported (Fine *et al.*, 1963; Kline and Clayton, 1964; Johnson and Kampschmidt, 1965). The hepatoma pattern was similar to that of liver. Rosado *et al.* (1969) did not find any correlation between hepatoma LDH patterns and growth rate and in all cases there was a predominance of M subunits; occasionally no H subunits were detected and in a few hepatomas the H subunit proportion was greater than in normal liver. Others have reported increases in the proportion of H subunit-containing isozymes in rat hepatomas (Kline and Clayton, 1964; Johnson and Kampschmidt, 1965; Schapira and de Néchaud, 1968). In one instance, an abnormal isozyme between LDH_2 and LDH_3 was detected in hepatoma and was also observed in foetal liver (Schapira and de Néchaud, 1968). In rat kidney and mammary tumours, however, increases in the proportion of M subunits have been observed (Rosado *et al.*, 1969; Schweitzer *et al.*, 1972). An increase in proportion of M subunits in hepatoma would not have been detected in this study since liver had mainly M subunits.

Relatively few studies of isozyme patterns in human hepatomas have been reported. PGM and G6PD isozyme patterns have not previously been examined. In an earlier study carried out by others in this laboratory (Balinsky *et al.*, 1973a), in which HK, PK and LDH isozyme patterns in human livers and hepatomas were compared, it was found that in hepatoma, liver-specific HK and PK isozymes were replaced by other forms and that there were occasional differences for LDH. In this work, PGM and G6PD isozyme patterns in muscle, liver and hepatoma were examined and HK, PK and LDH isozyme patterns in normal adult and foetal tissues, hepatoma and other tumour tissues were compared. As a result of improved separation of the isozymes, additional forms of HK (HK II_f) and PK (intermediate bands between PK L and PK M_2) were detected and further differences between normal or host liver and hepatoma patterns were apparent. The patterns

in hepatoma and other tumour tissues were sometimes found to resemble those in foetal tissues.

In human tissues, PGM and G6PD showed genetic variation. Many variants have been reported for these enzymes (Spencer *et al.*, 1964; Hopkinson and Harris, 1965, 1966; Kirkman, 1971; Beutler, 1972). Tissue differences in isozyme patterns were not apparent for PGM but were occasionally observed for G6PD.

As in rat, the human PGM patterns consisted of a series of multiple forms which probably result from oxidation of free sulphydryl groups (Greene and Dawson, 1973; Dawson and Greene, 1975). The patterns in normal muscle and liver were similar in tissues derived from the same individual. Spencer *et al.* (1964) did not observe any differences in the patterns in several tissues derived from the same individual. Since host livers and the corresponding hepatomas showed similar PGM patterns on electrophoresis and the Michaelis constants of this enzyme for glucose-1-phosphate were similar in these tissues, the reduced activity in human hepatoma (Balinsky *et al.*, 1973a) probably results from decreased enzyme protein synthesis, as in rat hepatoma.

In most cases the G6PD patterns in human host liver and hepatoma were similar; it was previously reported that the activities of this enzyme in host liver and hepatoma did not differ significantly (Balinsky *et al.*, 1973a). The faster-moving band present in two hepatomas, which had been stored frozen for some time, could have resulted from dissociation of the enzyme into the monomeric form, as has been suggested for the faster-moving band observed in red cells when electrophoresis was carried out using a discontinuous borate buffer system (Kirkman, 1968, 1971).
It was, however, interesting that in the surgical host liver and hepatoma,

which had a different G6PD variant, the hepatoma pattern differed from that of host liver; the significance of this is not clear at present.

The isozyme (HK II_f) of mobility intermediate between HK II and HK III was observed in human foetal muscle, liver, heart and brain, adult liver, hepatoma, meningioma and mesothelioma but not in adult muscle. This isozyme was first described by Benson and de Jong (1968) but was observed only in foetal liver. HK II_f stained heavily in the presence of 0.1 M glucose but hardly at all with 0.5 mM glucose, as reported by Benson and de Jong (1968). The value obtained, by others in this laboratory (Cayanis and Balinsky, 1975), for the Michaelis constant of HK II_f for glucose (0.07 mM) is not consistent with these staining characteristics and, at present, the reason for the discrepancy is not clear. It is not certain whether HK II_f is a separate isozyme or whether it represents a degradation product of one of the other isozymes. Splitting of rat HK II has been observed in the presence of trypsin or glucose (Grossbard and Schimke, 1966). Since HK II and HK II_f were both observed with DTT included in tissue extracts and electrophoresis buffers, it seems unlikely that HK II_f corresponds to HK II_a observed in rat adipose tissue and muscle (Katzen, 1966). Katzen (1966) showed that in the absence of mercaptoethanol in the electrophoresis buffers, HK II was split into a slower band (II_b) and a faster band (II_a), whereas in the presence of mercaptoethanol only HK II_a was observed. Shatton *et al.* (1969) have, however, occasionally observed splitting of rat HK II even in the presence of mercaptoethanol. Rogers *et al.* (1975) have reported splitting of human hexokinases I, II and III in liver, but did not include DTT or mercaptoethanol in the tissue extracts or electrophoresis buffers.

The tissue distributions of the HK isozymes in normal human muscle and liver differed from those in rat. Muscle had only HK I and

liver had HK I, HK II_f (a trace) and HK III. Neumann *et al.* (1974) found that human muscle had only HK I, except in one case when there was also HK II and Benson and de Jong (1968) and Rogers *et al.* (1975) reported that muscle had predominantly HK I and some HK II. Neumann *et al.* (1974) and Rogers *et al.* (1975) did not detect HK II in autopsy liver but others have reported presence of this isozyme in autopsy (Benson and de Jong, 1968) as well as in surgical tissue (Brown *et al.*, 1967). In the work described here, HK IV was not detected in normal human autopsy liver, which is in agreement with the findings of Lauris and Cahill (1966) and Neumann *et al.* (1974), but Brown *et al.* (1967) and Benson and de Jong (1968) reported the presence of this isozyme in surgical and autopsy material, respectively. HK IV was, however, detected on one occasion in this study, in a fresh host liver obtained during surgery but was unstable to freezing. The level of HK IV is controlled by diet (Sharma *et al.*, 1963; Niemeyer *et al.*, 1963) and its presence or absence is probably dependent on the dietary state of the individual from whom tissue is derived. It is also possible that, owing to greater lability of this isozyme, activity is lost during the time which elapses before electrophoresis is carried out.

The HK isozyme pattern in human foetal liver was similar to that reported by Benson and de Jong (1968). The proportion of HK II_f was greater than in adult liver and HK IA, which was detected only in foetal liver, has also been observed in foetal rat liver (Herzfeld and Greengard, 1972; Weinhouse *et al.*, 1972a, 1972b; Sigman *et al.*, 1972). Foetal muscle and heart had mainly HK I and traces of HK II and HK II_f, whereas Benson and de Jong (1968) observed only HK I in these tissues. Foetal brain had HK I and HK II_f whereas adult brain has been reported to have only HK I (Neumann *et al.*, 1974). Rogers *et al.* (1975) found that foetal muscle, heart and brain all had HK I and HK II.

There did not appear to be any age-related differences in the HK patterns in tissues of human foetuses ranging from 10 to 28 weeks gestation, hence the changes to adult patterns must occur at a later stage, perhaps at birth or after.

Rat host liver had a similar HK pattern to that of normal liver and did not appear to be affected by the tumour growing in the hind leg, whereas apparently uninvolved human host liver, which was probably representative of a precancerous state, showed trends similar to those occurring in the adjacent hepatoma, although to a lesser extent. In human hepatoma, as in the fairly fast-growing rat hepatoma, and as previously reported (Balinsky *et al.*, 1973a) the proportion of HK III decreased, while that of HK II increased. In addition, the results obtained in this study showed that HK II_f was also increased in the hepatoma. Although the isozyme pattern was altered, the activity of HK in hepatoma was not found to differ significantly from that of host liver (Balinsky *et al.*, 1973a). The increase in proportion of HK II and HK II_f in hepatoma suggests a trend towards the foetal liver pattern, which is consistent with the view that the foetal state tends to be reproduced in cancer (Schapira, 1973; Schapira *et al.*, 1975; Potter, 1969; Potter *et al.*, 1972).

Recent studies (Cayanis and Balinsky, 1975) showed that human HK I and HK III had lower Michaelis constants for glucose than HK II and HK II_f and that at high concentrations of glucose HK III was inhibited but the other isozymes were unaffected. This would suggest that in hepatoma, HK would not be inhibited at high concentrations of glucose and glucose could be used for glycolysis at all concentrations, whereas in normal liver, enzyme activity would be reduced at high glucose concentrations.

Normal dura had only HK I, whereas meningioma had HK I, HK II and

HK II_f, as did mesothelioma and this could suggest a trend towards a foetal pattern in these tumour tissues. Increases in HK II have also been reported in human cervical and corpus carcinomas (Kikuchi *et al.*, 1972; Sato *et al.*, 1972c). HK II was not, however, observed in the oesophageal cancer tissue examined in this work.

As reported by Imamura and Tanaka (1972), human muscle had PK M_1 and liver had PK L and only a trace of PK M_2. Bigley *et al.* (1968) and Osterman and Fritz (1973), however, did not distinguish clearly between the M_1 and M_2 isozymes but found that human liver had a substantial proportion of PK M.

The increased mobility of human PK M_1 in the presence of fructose-1,6- diphosphate in the gel and electrode buffers could be due either to a change in the conformational state of the isozyme or to an increase in negative charge as a result of binding with FDP. The relative mobilities of PK M_1 and PK M_2 in rat tissues have also been shown to change in the presence of FDP (Susor and Rutter, 1971; Osterman *et al.*, 1973). Whittell *et al.* (1973) showed that change of pH resulted in change in the relative mobilities of the rabbit PK M_1 and PK M_2 isozymes; at pH 7.0, PK M_1 was faster than PK M_2, but at pH 8.0, PK M_2 had the greater mobility.

Studies of PK isozyme patterns in human foetal tissues have not previously been reported. In this work it was found that patterns in human foetal liver and muscle were similar to the adult patterns, whereas in rat, adult patterns are acquired only after birth (Imamura and Tanaka, 1972). The predominant isozyme of human foetal liver was PK L, whereas foetal rat liver was found to have mainly PK M_2 (Imamura and Tanaka, 1972; Walker and Potter, 1972; Osterman *et al.*, 1973; Schapira *et al.*, 1975). The additional isozyme observed in some foetal livers, appears to have similar mobility to that reported for human erythrocyte PK (Nakashima, 1974;

Lincoln *et al.*, 1975) and presumably corresponds to the red cell isozyme of foetal haemopoietic tissue. An isozyme corresponding to the erythrocyte form has also been observed in foetal rat liver (Schapira and Gregori, 1971; Osterman *et al.*, 1973). It has been suggested that the erythrocyte isozyme may be an L-M_2 hybrid (Imamura and Tanaka, 1972; Whittell *et al.*, 1973) but recent studies have indicated that this is not the case (Lincoln *et al.*, 1975). Human foetal muscle and brain had PK M_1 and foetal heart had PK M_2 whereas Imamura and Tanaka (1972) found that foetal rat muscle and brain had mainly PK M_2. Imamura and Tanaka (1972) suggested that PK M_2 should be regarded as the prototype of PK isozymes and the L and M_1 forms as the differentiated types. It would be of interest to study the tissue patterns of smaller human foetuses to determine whether PK M_2 is the predominant isozyme at an earlier stage.

The PK patterns in rat host liver were unaffected by the tumour growing in the hind leg, whereas human host liver patterns were affected by the adjacent hepatoma and differed from those of normal liver. In most hepatomas, PK L was replaced either by PK M_2, as in the fairly fast-growing rat hepatoma, or by PK M_2 together with additional bands running between PK L and PK M_2. It was shown previously (Balinsky *et al.*, 1973a) that the increased activity of PK in human hepatoma was associated with the replacement of PK L by PK M, but the M_1 and M_2 isozymes were not separated and it is unlikely that intermediate forms would have been observed under the experimental conditions used.

Additional bands of electrophoretic mobility intermediate between PK L and PK M_2 have previously been observed in rat and mouse livers as well as in kidneys of several species, including human (Imamura and Tanaka, 1972; Whittell *et al.*, 1973; Osterman and Fritz, 1973; Cardenas *et al.*, 1975). The intermediate bands of bovine kidney have recently been shown to be hybrids of PK L and PK M_2 (Cardenas *et al.*, 1975). Crisp and Pogson (1972) and Van Berkel *et al.* (1972) showed that in normal rat liver PK L occurs

only in parenchymal cells and PK M_2 in the non-parenchymal tissue. PK M_2 however, occurs in foetal liver hepatocytes and can be switched on in regenerating rat liver parenchyma (Tanaka *et al.*, 1967; Walker and Potter, 1972). It is not certain whether the cellular localisation of these isozymes is the same in human as in rat, but it would appear from this study that in human hepatoma tissue, derived from liver parenchymal tissue, PK L and PK M_2 can occur concurrently and it is possible that the intermediate bands may be hybrids of PK L and PK M_2. However, attempts by the author to produce hybrids from the human PK L and M_2 isozymes, using the methods of Chilson *et al.* (1965) and Cardenas *et al.* (1975), have not so far been successful.

Since PK L is under hormonal and nutritional control (Krebs and Eggleston, 1965; Tanaka *et al.*, 1967), its loss in most hepatomas would lead to a loss of such control of enzyme activity. Since human hepatoma PK M has been shown to have a lower Michaelis constant for phosphoenolpyruvate than PK L (Balinsky *et al.*, 1973b), the increase in proportion of PK M in many of the hepatomas suggests that the enzyme would be active at both low and high phosphoenolpyruvate concentrations. Phosphoenolpyruvate could therefore be used for glycolysis at all concentrations, whereas in normal liver, at low concentrations, it could be diverted into the gluconeogenic pathway. This is consistent with the findings that the opposing gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, are lower in hepatoma than in normal or host liver indicating reduced gluconeogenesis in the tumour (this study).

Other human tumour tissues, meningioma, mesothelioma and oesophageal cancer all had PK M_2. An isozyme with slower mobility than PK M of liver or muscle has been observed in human lung, stomach and jejenum carcinomas and also in placenta (Spellman and Fottrell, 1973).

All five LDH isozymes were observed in human tissues. Normal muscle and liver had LDH_4 and LDH_5 as the major isozymes and the patterns were similar to those previously reported (Vesell and Bearn, 1961; Vesell, 1965).

The foetal tissues studied all had LDH_3 as the major isozyme and there was no variation in the patterns with foetal age, the redistribution of the isozymes presumably occurring at a later stage. This is consistent with the findings of Pfleiderer and Wachsmuth (1961). In chicken and rabbit, the LDH pattern of foetal muscle also differs from that of adult muscle but in rat and mouse, the foetal and adult muscle patterns are similar (Dawson *et al.*, 1964; Dawson and Kaplan, 1965). The foetal livers of most mammalian species, including human, have previously been shown to have a greater proportion of H subunits than adult livers (Pfleiderer and Wachsmuth, 1961; Vesell *et al.*, 1962; Fine *et al.*, 1963).

The LDH isozyme patterns of most of the human host livers and hepatomas were similar to the normal liver pattern, but occasionally an increase in the proportion of H subunits was observed in hepatoma, which is consistent with the observations of others in this laboratory (Balinsky *et al.*, 1973a). It was also reported previously that the activity of LDH was decreased in hepatoma (Balinsky *et al.*, 1973a) and it would appear that, in the majority of cases, this results from decreased enzyme protein synthesis. It was apparent from this study that in cases where the proportion of H subunits was increased in hepatoma, the hepatoma pattern resembled that of foetal liver. Others have occasionally reported increases in proportion of the H form, but in the majority of non-hepatic human tumours increases in the proportion of the M form were observed (Starkweather and Schoch, 1962; Goldman and Kaplan, 1963; Goldman *et al.*, 1964; Langvad, 1972). The significance of the increase in the H form in some tumours

is not at present clear. Increases in proportion of M subunits could be related to the anaerobic conditions prevailing in tumour tissues.

Meningioma, mesothelioma and oesophageal cancer tissue have not previously been studied, and all were found to have a high proportion of H subunits.

Although species-specific isozyme mobilities were maintained in the rat and human cell cultures, the distributions of the various isozymes differed somewhat from those of the tissue of origin. Tissue-specific isozyme patterns are frequently lost in culture, presumably as a result of adaptation of the cells to culture conditions (Nitowsky and Soderman, 1964; Vesell, 1965; Katzen *et al.*, 1965; Montes de Oca *et al.*, 1969; Walker *et al.*, 1972). The complex structural and biochemical interactions *in vivo* are not maintained *in vitro* and the *in vitro* conditions may favour the formation of particular isozymes. It is also possible that selection occurs during the early stages of culture, for a cell containing isozymes which would give it an advantage for cell division.

In the rat and human hepatoma cells there was a greater proportion of HK II than in the respective tumours and HK II was detected in human oesophageal cancer cells but not in oesophageal cancer tissue. This increase or appearance of HK II could be related to the glucose content of the culture medium, since Katzen *et al.* (1965) showed that in human liver cells, HK II increased in the presence of high concentrations of glucose. The presence of the liver-specific isozyme HK IV in the rat hepatoma cells and not in hepatoma, may also be related to the glucose concentration of the medium. Yoshida ascites hepatoma cells and rat and human liver cells were found to have HK isozymes I and II, (Katzen *et al.*, 1965; Sato *et al.*, 1972b; Walker *et al.*, 1972) which is as would be expected for hepatoma

tissue, but HK I and HK III are the forms found in liver tissue.

The cell cultures all had PK M_2, as did rat hepatoma tissue and human oesophageal cancer tissue, but the original tumour from which human hepatoma cells (A) were derived appeared to have the M_1 isozyme. Walker *et al.* (1972) found that normal rat liver cells had PK M_2 and that the kinetic properties of this isozyme varied with culture conditions.

The LDH patterns in rat hepatoma cells and human oesophageal cancer cells were similar to those of the corresponding tissues, but there was an increase in the proportion of H subunits in human hepatoma cells. German *et al.* (1964) found that there was an increase in the proportion of H subunits when HeLa cells were grown in the absence of serum. Others have reported a high proportion of M subunits in a wide variety of cultures (Nitowsky and Soderman, 1964; Vesell, 1965). Fondy and Kaplan (1965) found that decreasing the oxygen tension during growth resulted in an increase in the proportion of M subunits in cell cultures derived from mouse, chicken and monkey.

The isozyme patterns in the cell cultures were not truly representative of those of the tissues of origin but were, in general, characteristic of the patterns usually acquired by rapidly-dividing rat or human cells growing in culture. Non-dividing cells derived from rat liver have, however, been reported to retain tissue-specific isozyme patterns (Bonney *et al.*, 1973).

The isozyme patterns obtained using polyacrylamide disc gel isoelectric focusing were, in general, more variable and more complex than those obtained using starch gel electrophoresis and in order to correlate the pI values with the electrophoretic mobilities of the various isozymes, further extensive studies would be necessary.

Rat and human PGM patterns consisted of a series of bands which could correspond to the multiple forms observed on starch gel electrophoresis. Harshman and Six (1969) separated four forms of rabbit muscle PGM by isoelectric focusing in a sucrose density gradient but Greene and Dawson (1973) using the same technique did not achieve a clear separation of the rabbit muscle isozymes and found that successive fractions contained mixtures of the starch gel electrophoretic forms. As on starch gel electrophoresis, hepatoma patterns were similar to host liver patterns.

Isoelectric focusing of rat hepatoma extracts showed an increase in intensity of the more cathodic HK bands, as compared with normal or host liver and it would therefore seem likely that these bands correspond to HK II, which was shown to increase in hepatoma. An isozyme corresponding to HK IV which stains only in the presence of high glucose on starch gel electrophoresis was not observed on isoelectric focusing, possibly because it was unstable under the experimental conditions.

The major cathodic band of LDH activity (pH 8.3 - 9.0) observed in rat liver and hepatoma, presumably corresponds to LDH_5 for which a pI of 8.6 has previously been reported (Weller *et al.*, 1973). In addition, bands in the pH region 4.8 - 6.8 were observed in both tissues but the number and intensity of these bands was increased in hepatoma. By means of isoelectric focusing in a sucrose density gradient, Fukuda *et al.* (1973) and Katayama (1974) found that the proportion of the isozyme of pI 6.5 increased in rat hepatoma and that the hepatoma pattern was similar to the foetal liver pattern.

Isoelectric focusing of extracts of human tissues which had been frozen gave rise to as many as twelve bands of LDH activity. Others have also observed a greater number of bands in human tissue extracts and

serum on gel isoelectric focusing than on electrophoresis (Latner, 1973; Fukuda *et al.*, 1973); it has been suggested that the additional bands could be attributed to the formation of enzyme complexes with Ampholines or with tissue proteins (Latner, 1973). However, in fresh human liver only the most cathodic band, presumably corresponding to LDH_5, which has been shown to have a pI value 9.4 (Weller *et al.*, 1973), was detected in this study. In fresh human hepatoma, two or three additional anodic bands were detected and these probably represent an increase in the proportion of H subunits as was occasionally observed on starch gel electrophoresis. Fukuda *et al.* (1973) have shown increases in the number of bands in the alkaline and neutral pH regions for a variety of cancer sera.

Isozymes are numerous and patterns often vary from species to species and from tissue to tissue, depending on the degree of differentiation and the specific functions associated with a particular tissue. This diversity was apparent for the various isozyme systems considered in this study. Since normal adult tissues of a particular species have characteristic isozyme patterns, studies of isozyme changes in hepatomas and other tumours provide information concerning the effects of the malignant transformation. The results of this study showed that, although the tissue distributions of the isozymes of the key glycolytic enzymes HK and PK often differed in rat and human, in human hepatoma, as in rat hepatoma, liver-specific isozymes were replaced by other forms, some of which occur in foetal tissue. Loss of these isozymes suggests a loss of the differentiated functions with which they are associated in normal tissue; the isozymes occurring in hepatoma are probably those associated with energy production for increased cell growth and division. Since differentiated functions may not be present in foetal tissues, it is not surprising to find some resemblance between foetal

and tumour tissues. Schapira (1966) suggested that in cancer, synthesis of adult forms of enzymes was repressed while that of embryonic forms was derepressed and Potter (1969) put forward a similar but more restrictive hypothesis of 'oncogeny as blocked ontogeny'.

Since relatively few isozyme studies have been carried out on human tumours, examination of isozyme systems of other key enzymes could provide further knowledge of the metabolic processes occurring in human cancerous tissues.

CHAPTER III

PURIFICATION AND PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM MONKEY LIVER

SUMMARY

1. Phosphoenolpyruvate carboxykinase (PEPCK) was found to be almost equally distributed between the cytosol (42.5%) and mitochondrial fractions of monkey liver (47.7%).

2. PEPCK was purified from the cytosol fraction of monkey liver by means of ammonium sulphate fractionation, Sephadex G-100, DEAE-Sephadex A-50, hydroxylapatite and cellulose phosphate P11 chromatography. The enzyme was purified from monkey liver mitochondria by means of DEAE-Sephadex A-50, Sephadex G-200 and hydroxylapatite chromatography.

3. Two forms of PEPCK (a major form and a minor form which were separated on DEAE-Sephadex A-50) were obtained from the cytosol fraction and a single form from the mitochondria. The minor form occurring in the cytosol, however, resembled the mitochondrial enzyme in all respects studied and it therefore seemed most probable that this was in fact the mitochondrial enzyme, which was present in the cytosol as a result of mitochondrial leakage during the subcellular fractionation procedure.

4. The cytosol and mitochondrial enzymes were purified approximately 130-fold and had final specific activities of 3.3 and 14.6 U/mg, respectively.

5. The enzymes obtained from the cytosol and mitochondria had similar molecular weights, which were of the order 66,000.

6. Kinetic studies of the carboxylation and decarboxylation reactions were carried out and a comparison of the properties of the purified cytosol and mitochondrial enzymes was made. The two enzymes were similar in a number of respects. Some differences were, however, apparent suggesting that the two forms are distinct proteins.

7. When assayed in the carboxylation direction, cytosol PEPCK was more active with GDP than with IDP, whereas the mitochondrial form was equally active with both substrates. Neither form showed significant activity with ADP, CDP or UDP. When assayed in the decarboxylation direction, both forms were more active with ITP than with GTP or UTP and showed only slight activity with ATP. With CTP, cytosol PEPCK showed some activity whereas the mitochondrial form was inactive.

8. For the carboxylation reaction, the cytosol and mitochondrial enzymes had similar pH optima (pH 6.8 and 7.0, respectively) whereas for the decarboxylation reaction, the cytosol enzyme had a broader pH optimum (pH 7.0 - 8.0) than the mitochondrial form (pH 7.0 - 7.2).

9. When assayed in either direction, both enzymes were more active with manganese than with magnesium, although the activity with magnesium, relative to that with manganese, was lower for the cytosol enzyme than for the mitochondrial enzyme. In the carboxylation direction, both enzymes were less active with iron II than with manganese.

10. The effects of varying concentrations of manganese at different concentrations of nucleoside phosphate were similar for both enzymes. In the carboxylation direction, using 0.04, 1, 2 and 4 mM IDP, maximal activities were obtained with manganese chloride : IDP ratios of the order 30:1, 2:1, 2:1 and 1:1, respectively. The ratios

manganese chloride : GDP required for maximal activity were of the order 100:1 and 2:1 for 0.005 and 1 mM GDP, respectively. There was only slight inhibition at manganese chloride concentrations exceeding those required for maximal activity. In the decarboxylation direction, using 0.2, 1, 2 or 4 mM ITP or 0.5 or 2 mM GTP, it was found that the activities of both enzymes reached a maximum when the concentration of manganese chloride was equal to that of ITP or GTP and then decreased as the manganese chloride concentration was increased further.

11. In the direction of oxaloacetate synthesis, the cytosol and mitochondrial enzymes had similar Michaelis constants for phosphoenolpyruvate (0.14 mM and 0.15 mM, respectively) as well as for sodium bicarbonate (9.7 mM and 12.5 mM, respectively). The Michaelis constants of the cytosol enzyme for IDP (0.29 mM) and GDP (0.033 mM) were, however, somewhat higher than those of the mitochondrial enzyme (0.12 mM and 0.014 mM, respectively). In the direction of phosphoenolpyruvate synthesis, the cytosol and mitochondrial enzymes had similar Michaelis constants for oxaloacetate (0.027 mM and 0.022 mM, respectively) as well as for GTP (0.16 mM and 0.13 mM, respectively). The Michaelis constant of cytosol PEPCK for ITP (1.1 mM) was higher than that of mitochondrial PEPCK (0.32 mM).

12. The activities of the two enzymes were not significantly affected by the presence of AMP, amino acids, α-ketoglutarate, succinate, malate or sodium fluoride when assayed in either direction. In the carboxylation direction, hydrazine sulphate had little effect whereas IMP, ITP, GTP, citrate, isocitrate and quinolinic acid inhibited both enzymes. The effects of ITP, GTP, citrate and quinolinic acid were, however, considerably less marked when iron II replaced manganese as the metal-ion activator. In the presence

of iron II, the mitochondrial enzyme was somewhat inhibited by tryptophan whereas the cytosol form was unaffected. In the decarboxylation direction, GDP, citrate, isocitrate and quinolinic acid inhibited both enzymes but the cytosol form was affected to a slightly greater extent than the mitochondrial form. IDP and hydrazine sulphate inhibited cytosol PEPCK, but had little effect on the mitochondrial form.

METHODS

Chemicals

Substrates, cofactors and enzymes

Monosodium phosphoenolpyruvate (PEP), oxaloacetic acid (OAA), trilithium GDP, disodium ADP, disodium ATP, disodium AMP, disodium IMP, disodium NADP, α-ketoglutaric acid, trisodium glycerate-2-phosphate and the enzymes malate dehydrogenase (MDH), lactate dehydrogenase (LDH), pyruvate kinase (PK), hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD), aldolase and enolase were obtained from Biochemica Boehringer, Mannheim, Germany; disodium IDP, trisodium ITP, trisodium GTP, sodium UDP, sodium UTP, sodium CDP, sodium CTP and disodium NADH from Sigma Chemical Co., St. Louis, Missouri, U.S.A. and sodium bicarbonate-^{14}C from the Radiochemical Centre, Amersham, England.

Other chemicals

Dithiothreitol (DTT) was obtained from Calbiochem, San Diego, California, U.S.A.; glutathione (reduced form) (GSH) from Sigma; bovine plasma albumin from Armour Pharmaceutical Co., Eastbourne, England; cytochrome c from British Drug Houses, Poole, England and haemoglobin from Hycel Inc., Houston, Texas, U.S.A. DEAE-Sephadex A-50, Sephadex G-75, Sephadex G-100, Sephadex G-200 and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden. Hydroxylapatite (dry powder) was from Calbiochem; cellulose phosphate P11 from Whatman Biochemicals, Maidstone, England and Carbowax (polyethylene glycol 20 M) from British Drug Houses. Other standard chemicals were obtained from British Drug Houses, Calbiochem, E. Merck, Darmstadt, Germany and Hopkin and Williams, Chadwell Heath, England and were of analytical grade.

Tissue

Livers of vervet monkeys, *Cercopithecus aethiops* (sub-species *pygerythrus*), were supplied by Dr. P.A.D. Winter. The monkeys, which had been fed a normal laboratory diet and had not been starved, were killed by Nembutal injection. The livers were removed, kept on ice and used as soon as possible.

SUBSTRATE ASSAYS

Concentrations of substrate solutions used in the phosphoenolpyruvate carboxykinase-catalysed reaction were determined spectrophotometrically by measuring oxidation of NADH or reduction of $NADP^+$ at 340 nm, using a Beckman DB spectrophotometer. Cuvettes of capacity 3 ml were used.

Phosphoenolpyruvate (PEP)

The method, which was similar to that described by Czok and Eckert (1965), was based on the reactions shown below.

$$\text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

The reaction mixture contained 50 mM triethanolamine - 5 mM EDTA - 10 mM magnesium chloride, pH 7.4, 2.9 mM ADP, 0.15 mM NADH, 4 U pyruvate kinase, 4 U lactate dehydrogenase and PEP solution. The optical density of the mixture was noted in the absence of pyruvate kinase; pyruvate kinase was then added and the change in optical density on completion of the reaction was determined.

Inosine diphosphate (IDP) and guanosine diphosphate (GDP)

The method, which was similar to that described by Adam (1965), was based on the pyruvate kinase and lactate dehydrogenase reactions shown above, with IDP or GDP replacing ADP.

The reaction mixture contained 50 mM triethanolamine - 5 mM EDTA - 10 mM magnesium chloride, pH 7.4, 3 mM phosphoenolpyruvate, 0.15 mM NADH, 12 U pyruvate kinase, 4 U lactate dehydrogenase and IDP or GDP solution. The optical density of the mixture was noted in the absence of pyruvate kinase; pyruvate kinase was then added and the change in optical density on completion of the reaction was determined.

Oxaloacetate (OAA)

The method was similar to that of Hohorst and Reim (1965).

$$\text{OAA} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$$

The reaction mixture contained 100 mM imidazole - HCl, pH 7.0, 0.15 mM NADH, 4 U malate dehydrogenase and oxaloacetate solution. The optical density of the mixture was noted in the absence of malate dehydrogenase; malate dehydrogenase was then added and the change in optical density on completion of the reaction was determined.

Inosine triphosphate (ITP) and guanosine triphosphate (GTP)

The method, which was similar to that of Lamprecht and Trautschold (1965) for ATP, was based on the reactions shown below.

$$\text{glucose} + \text{ITP (or GTP)} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{IDP (or GDP)}$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

The reaction mixture contained 50 mM triethanolamine - 5 mM EDTA - 10 mM magnesium chloride, pH 7.4, 1.5 mM glucose, 0.36 mM $NADP^+$, 4 U hexokinase, 4 U glucose-6-phosphate dehydrogenase and ITP or GTP solution. The optical density of the mixture was noted in the absence of hexokinase; hexokinase was then added and the change in optical density on completion of the reaction was determined.

ENZYME ASSAYS

All assays were carried out at 30°. Spectrophotometric assays were carried out in 3 ml cuvettes using a Beckman DB spectrophotometer. In all cases, reaction rates were linear with respect to time and amount of enzyme at the concentrations used.

Phosphoenolpyruvate carboxykinase (PEPCK)

Phosphoenolpyruvate carboxykinase catalyses the reversible reaction shown below.

$$PEP + NDP + CO_2 \underset{PEPCK}{\overset{Metal^{2+}}{\rightleftharpoons}} \text{oxaloacetate} + NTP$$

Enzyme activity was measured either in the direction of oxaloacetate synthesis (carboxylation reaction), or in the physiological direction of phosphoenolpyruvate synthesis (decarboxylation reaction).

Carboxylation assays

Radioactive method

This assay was used during subcellular fractionation experiments and throughout the purification procedure. The method was that of Chang and Lane (1966), as modified by Ballard and Hanson (1967). Incorporation of ^{14}C-bicarbonate into malate was measured and the assay was carried out as

described in Chapter I (p. 85).

Continuous spectrophotometric method

This assay was used only for purified enzyme preparations which were free from pyruvate kinase activity. As in the radioactive method, the PEPCK reaction was coupled to the malate dehydrogenase reaction. The overall reaction rate was determined by following NADH oxidation at 340 nm.

All solutions were degassed before use and stored over soda lime to minimise the content of dissolved carbon dioxide. The assay mixture, which was similar to that described by Chang and Lane (1966), contained 100 mM imidazole - HCl, pH 6.6, 2 mM manganese chloride, 1 mM glutathione (reduced form), 1.25 mM IDP, 50 mM sodium bicarbonate, 3 mM phosphoenol-pyruvate, 0.15 mM NADH, 4 U malate dehydrogenase and 0.1 ml of suitably diluted enzyme. The total volume was 3 ml and the final pH was 6.9 - 7.1 at 30°. After a 3 minute preincubation period (to eliminate a slight lag), the reaction was initiated by addition of IDP. IDP was omitted from the blank.

Decarboxylation assays

Seubert-Huth method

The assay was based on that of Seubert and Huth (1965) and was similar to that described by Ballard (1970). The incubation medium contained 100 mM imidazole - HCl, pH 7.0, 1 mM glutathione (reduced form), 5 mM manganese chloride, 1 mM oxaloacetate, 5 mM ITP and 0.1 ml of suitably diluted enzyme in a total volume of 1 ml. The reaction was initiated by addition of oxaloacetate. Incubation times ranged from 4 to 10 minutes depending on enzyme activity. Reactions were stopped by addition of 10 mg solid sodium borohydride, the assay tubes were placed in an ice bath

for 2 minutes and excess sodium borohydride was then destroyed by addition of 0.2 ml 15% perchloric acid. The mixtures were neutralised by addition of 0.2 ml 3 M potassium bicarbonate and then centrifuged at 50 g, at room temperature, for 5 minutes. Phosphoenolpyruvate formed during the reaction was determined in an aliquot (0.5 - 1 ml) of the supernate using the method described on p.165.

There was no significant blank reaction in the absence of either oxaloacetate or ITP.

Continuous spectrophotometric method

This assay was used only for purified enzyme preparations which were not contaminated with malate dehydrogenase. The method was similar to that described by Chang and Lane (1966). The PEPCK reaction was coupled to the PK and LDH-catalysed reactions (shown on p.165) and the overall rate was determined by following NADH oxidation at 340 nm.

The assay mixture contained 100 mM imidazole-HCl, pH 7.0, 5 mM ITP, 0.5 mM oxaloacetate, 5 mM manganese chloride, 2.9 mM ADP, 0.15 mM NADH, 1 mM glutathione (reduced form), 4 U pyruvate kinase, 4 U lactate dehydrogenase and 0.1 ml of suitably diluted enzyme in a total volume of 3 ml. The reaction was initiated by addition of oxaloacetate to both cuvettes. Enzyme was omitted from the blank. In the absence of enzyme, there was a blank reaction due to the non-enzymic breakdown of oxaloacetate to pyruvate; this reaction decreased with decreasing oxaloacetate concentration. The rate of non-enzymic breakdown of oxaloacetate increased in the absence of ITP, presumably because more manganese was then available to stimulate the reaction. Blanks from which oxaloacetate or ITP were omitted were therefore unsuitable.

Marker enzymes

During subcellular fractionation, glutamate dehydrogenase and lactate dehydrogenase were used as marker enzymes for the mitochondrial and cytosol fractions, respectively.

Glutamate dehydrogenase (GDH)

Glutamate dehydrogenase activity was determined using a continuous spectrophotometric assay, similar to that described by Delbrück *et al.* (1959), in which the rate of oxidation of NADH was followed at 340 nm.

$$\alpha\text{-ketoglutarate} + NH_3 + H^+ + NADH \xrightarrow{GDH} \text{glutamate} + H_2O + NAD^+$$

The reaction mixture contained 50 mM Tris-HCl-1 mM EDTA, pH 8.0, 3 mM α-ketoglutarate, 40 mM ammonium acetate, 0.15 mM NADH and 0.1 ml of suitably diluted enzyme in a total volume of 3 ml. The reaction was initiated by addition of α-ketoglutarate. α-Ketoglutarate was omitted from the blank.

Lactate dehydrogenase

Lactate dehydrogenase activity was determined using the method described in Chapter II (p.120).

Interfering enzymes

The activities of enzymes which could interfere during kinetic studies were assayed in PEPCK preparations at various stages of purification. The assays were carried out under conditions similar to those used for PEPCK assays.

Pyruvate kinase

A continuous spectrophotometric method, based on that described by Shonk and Boxer (1964), was used. The pyruvate kinase reaction was coupled to the lactate dehydrogenase reaction (as shown on p.165) and oxidation of NADH was followed at 340 nm.

The reaction mixture contained 100 mM imidazole - HCl, pH 7.0, 3 mM phosphoenolpyruvate, 1.25 mM IDP, 2 mM manganese chloride, 0.15 mM NADH, 4 U lactate dehydrogenase and 0.1 ml of suitably diluted enzyme in a total volume of 3 ml. After a 5 minute preincubation period, the reaction was initiated by addition of IDP. IDP was omitted from the blank.

Enolase

A continuous spectrophotometric method, based on that described by Shonk and Boxer (1964), was used.

$$\text{2-phosphoglycerate} \xrightleftharpoons{\text{enolase}} \text{PEP} + H_2O$$

The enolase reaction was coupled to the pyruvate kinase and lactate dehydrogenase reactions (shown on p.165) and NADH oxidation was followed at 340 nm.

The reaction mixture contained 100 mM imidazole - HCl, pH 7.0, 2.8 mM 2-phosphoglycerate, 1.25 mM IDP, 2 mM manganese chloride, 0.15 mM NADH, 4 U pyruvate kinase, 4 U lactate dehydrogenase and 0.1 ml of suitably diluted enzyme in a total volume of 3 ml. The reaction was initiated by addition of 2-phosphoglycerate. 2-Phosphoglycerate was omitted from the blank.

Phosphatases ('IDPase' and 'ITPase')

The method used was based on that described for adenosine

triphosphatase (Lowry, 1957). The reaction mixture contained 100 mM imidazole - HCl, pH 7.0 and 1.25 mM IDP or 5 mM ITP. The reaction was initiated by addition of suitably diluted enzyme. The total volume of the reaction mixture was 1 ml.

Incubation times ranged from 5 - 60 minutes. Reactions were stopped by addition of 0.5 ml 10% trichloroacetic acid and precipitated protein was removed by centrifugation at 50 g, at room temperature, for 5 minutes. Inorganic phosphate formed during the reaction was determined in an aliquot (0.5 ml) of the supernatant as described below.

Anhydrous disodium hydrogen orthophosphate was used to prepare a series of standards. Molybdate reagent was prepared by mixing 0.1 M acetic acid - 0.065 M sodium acetate, 2.5% ammonium molybdate and 1% ascorbic acid in the proportion 46:2:2. Standards and samples of volume 0.5 ml were mixed with 5 ml of molybdate reagent and after 15 - 45 minutes optical density was measured at 870 nm.

Zero time (blank) values (enzyme was added after the trichloroacetic acid) were subtracted from the assay values.

Malate dehydrogenase

A continuous spectrophotometric method, based on that of Bergmeyer and Bernt (1965), was used. The rate of oxidation of NADH during the reaction (shown on p.166) was followed at 340 nm.

The reaction mixture contained 100 mM imidazole - HCl, pH 7.0, 0.3 mM oxaloacetate, 0.15 mM NADH and 0.1 ml of suitably diluted enzyme in a total volume of 3 ml. The reaction was initiated by addition of enzyme. Enzyme was omitted from the blank.

Enzyme activity

A unit of enzyme activity is defined as the amount of enzyme catalysing the conversion of 1 μmole of substrate to product per minute at 30°, under the conditions specified above.

PROTEIN DETERMINATION

During the subcellular fractionation procedure, proteins were determined using the method of Lowry *et al.* (1951), as described previously (p. 90).

Throughout the purification procedure, proteins were determined using the method of Warburg and Christian (1942) in which optical densities at 260 and 280 nm were measured and a correction applied to account for nucleic acid concentration.

SUBCELLULAR FRACTIONATION

The procedure used was based on the methods described by Mahler and Cordes (1966) and Johnson and Lardy (1967).

Centrifugation at 50 g, 600 g, and 15,000 g was carried out using a Sorvall SS-3 centrifuge which was kept in the cold room and maintained at 4°. Centrifugation at 100,000 g was carried out at 4°, using an MSE 25 refrigerated centrifuge.

Monkey liver (5 g) was cut into small pieces and homogenised in four volumes of ice-cold buffered sucrose medium, containing 20 mM triethanolamine, 0.25 M sucrose, 1 mM EDTA and 1 mM glutathione (reduced form) at pH 7.5, using 5 - 6 strokes of a Potter-Elvehjem homogeniser which had a loosely-fitting glass pestle. The use of either a motor-driven Potter-Elvehjem homogeniser with a Teflon pestle or a Waring blender

disrupted the mitochondria (as indicated by the amount of glutamate dehydrogenase activity in the cytosol) and did not give satisfactory fractionations.

The homogenate was centrifuged at 50 g for 3 minutes. The residue (unbroken cells) was washed twice by resuspending in the buffered sucrose medium and recentrifuging as before.

The combined 50 g supernatant and washings were centrifuged at 600 g for 5 minutes. The residue (nuclear fraction) was washed twice by resuspending in buffered sucrose medium and recentrifuging as before.

The combined 600 g supernatant and washings were centrifuged at 15,000 g for 20 minutes. The residue (mitochondrial fraction) was washed twice by resuspending in buffered sucrose medium and recentrifuging as before.

The combined 15,000 g supernatant and washings were centrifuged at 100,000 g for 30 minutes. The residue (microsomal fraction) was washed twice by resuspending in buffered sucrose medium and recentrifuging. The washings were combined with the 100,000 g supernatant (cytosol fraction).

Extracts were prepared from the particulate fractions using either the buffered sucrose medium or 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT, by freezing and thawing four times in an acetone-dry ice mixture. After centrifugation at 15,000 g (unbroken cells, nuclei and mitochondria) or 100,000 g (microsomes) for 30 minutes, the activities of phosphoenol-pyruvate carboxykinase, glutamate dehydrogenase and lactate dehydrogenase were determined in the supernatants obtained as well as in the cytosol fraction. Enzyme activity in the combined 50 g supernatant and washings, from which unbroken cells had been removed, was taken as the total activity of the homogenate and the recovered activity was the sum of the activities

in the subcellular fractions.

PURIFICATION PROCEDURES

Phosphoenolpyruvate carboxykinase (PEPCK) was purified from the mitochondrial and cytosol fractions of monkey liver. Subcellular fractionation was carried out as described above, using 40 - 60 g of liver. Glutamate dehydrogenase and lactate dehydrogenase were used as marker enzymes.

All steps in the purification were carried out at 4°.

The percentages of ammonium sulphate refer to saturation at 0° (100% saturation = 70 g ammonium sulphate/100 ml of solution). In all cases where ammonium sulphate was used, the suspensions were stirred gently for 30 minutes and precipitated protein was then collected by centrifugation at 31,000 g, at 4°, for 20 minutes, using an MSE 18 High Speed refrigerated centrifuge.

DEAE-Sephadex A-50, Sephadex G-100, Sephadex G-200 and hydroxylapatite were equilibrated with the appropriate buffer for 3 - 4 days (with at least three changes of buffer during this time) and then washed extensively on a Buchner funnel with the same buffer. Cellulose phosphate P11 was prepared according to the method of Thuma *et al.* (1972). It was washed on a Buchner funnel with 0.5 M sodium hydroxide - 0.5 M sodium chloride, rinsed with distilled water and then washed with 0.1 M hydrochloric acid, before being equilibrated with starting buffer.

Packed columns were equilibrated with at least two volumes of starting buffer. Eluates from columns were passed through an LKB 6250 H Uvicord absorptiometer (attached to a chopper bar recorder), which gave an optical density reading at 254 nm, and then collected using an LKB 7000 fraction

collector.

The salt concentrations at which enzyme eluted from the columns were estimated from conductivity measurements using a Radiometer conductivity meter.

Purification of cytosol phosphoenolpyruvate carboxykinase

The procedures used were similar to those described by Ballard and Hanson (1969) and Philippidis *et al.* (1972) for rat liver cytosol PEPCK, except that where these workers used DEAE-cellulose, DEAE-Sephadex was used in this study. In addition, a cellulose phosphate column was included as the final step.

Ammonium sulphate fractionation (30 - 55%)

Solid ammonium sulphate was added to the cytosol fraction (200 - 280 ml) to bring the solution to 30% saturation. The protein precipitate was discarded. Additional solid ammonium sulphate was added to increase the percentage saturation of the solution to 55%. The protein precipitate was collected and dissolved in the minimum volume of 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT.

Initially, in order to determine which ammonium sulphate fractions contained enzyme activity, the solution was brought to 25% saturation with solid ammonium sulphate and the percentage saturation was then increased in steps of 5% up to 60% saturation. Enzyme activity was assayed in successive fractions and as shown below, most of the activity precipitated in the fractions between 30 and 55% saturation.

Fraction	% Activity	Fraction	% Activity
0 - 25%	1.1	45 - 50%	20.8
25 - 30%	1.9	50 - 55%	8.3
30 - 35%	10.9	55 - 60%	0.3
35 - 40%	25.1	60% supernate	0
40 - 45%	31.6		

Sephadex G-100 chromatography

After ammonium sulphate fractionation, the enzyme (30 - 40 ml) was applied to a Sephadex G-100 column (19.6 cm^2 x 40 cm) which had been equilibrated with 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT. The enzyme was eluted with the same buffer at a flow rate of 2.5 ml/minute. Fractions of volume 8 ml were collected and the active fractions were combined.

The use of a Sephadex G-75 column of the same dimensions also proved satisfactory at this stage and the purification obtained was similar to that using a G-100 column.

DEAE-Sephadex A-50 chromatography

The Sephadex G-100 eluate (150 - 200 ml) was applied to a DEAE-Sephadex column (9.6 cm^2 x 15 cm) which had been equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed with the same buffer until all the unadsorbed protein had been removed. Elution was carried out firstly with 800 ml of a linear gradient of 0 - 0.15 M sodium chloride in 50 mM Tris-HCl, pH 8.0 and then with 400 ml of a linear gradient of 0.15 - 0.375 M sodium chloride in 50 mM Tris-HCl, pH 8.0. The flow rate was 1.5 ml/minute and the volume of each fraction collected was 9 ml.

Two peaks of enzyme activity were obtained, a minor peak which eluted on application of the first gradient, at a sodium chloride

concentration of about 0.08 M, and a major peak which eluted on application of the second gradient, at a sodium chloride concentration of about 0.15 M.

The enzyme was stable on the column, but lost activity rapidly if the fractions were not concentrated immediately. The use of two separate gradients was preferable to the use of a single gradient as the major form could be collected over a shorter period of time in a smaller volume. In order to separate the two peaks using a single gradient, a volume of 1,600 ml of linear gradient of 0-0.375 M sodium chloride in 50 mM Tris-HCl, pH 8.0 was required. When chromatography was carried out using 50 mM Tris-HCl, pH 7.0, the two peaks were not clearly separated.

The active fractions corresponding to each of the two peaks were combined separately and the solutions were brought to 70% saturation with solid ammonium sulphate. The protein precipitates were collected and dissolved in the minimum volume of 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT.

The concentrated enzymes were stable and could be stored at -20° for up to 2 months without loss of activity.

Further purification of the major cytosol enzyme

Hydroxylapatite chromatography

The concentrated DEAE-Sephadex eluate (8 - 10 ml) was dialysed against 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT for five hours. The dialysed enzyme was then applied to a hydroxylapatite column (9.6 cm^2 x 6 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was washed with the same buffer until all the unadsorbed protein had been removed. Elution was then carried out using 200 ml of a linear gradient of 0.01 - 0.25 M potassium phosphate buffer, pH 7.0 at a flow rate of 0.2 ml/minute. The volume of each fraction collected was 5 ml. The enzyme eluted at a potassium phosphate concentration of about 0.1 M. The

active fractions were combined and after addition of 1 mM DTT, the eluate was concentrated using an Amicon ultrafiltration cell with a PM10 membrane.

Cellulose phosphate P11 chromatography

The method was similar to that described by Thuma *et al.* (1972) for adenylate kinase. The concentrated hydroxylapatite eluate (5 - 8 ml) was dialysed against 10 mM imidazole-HCl, pH 7.0 containing 0.25 M sucrose and 1 mM DTT for 4 - 5 hours. The dialysed enzyme was then applied to a cellulose phosphate P11 column (9.6 cm^2 x 8 cm) which had been equilibrated with 10 mM imidazole-HCl, pH 7.0 containing 0.25 M sucrose and 1 mM DTT. The column was washed with the same buffer until all the unadsorbed protein had been removed. The enzyme was then eluted using 10 mM imidazole-HCl, pH 7.0 containing 0.25 M sucrose, 1 mM DTT and 0.2 M sodium chloride, at a flow rate of 1.6 ml/minute. The volume of each fraction collected was 3.5 ml. The active fractions were combined and the eluate was concentrated using an Amicon ultrafiltration cell with a PM10 membrane. The concentrated enzyme was stored in the deep freeze at -20°.

The enzyme was found to be extremely unstable if cellulose phosphate chromatography was carried out in the absence of sucrose in the buffer. In the absence of sucrose, 70 - 80% of the initial activity applied to the column was lost during the procedure.

Further purification of the minor form of PEPCK occurring in the cytosol

Hydroxylapatite chromatography

The concentrated DEAE-Sephadex eluate (3 - 5 ml) was dialysed against 10 mM potassium phosphate buffer, pH 7.0 for 4 - 5 hours. Hydroxylapatite chromatography was then carried out as described above for the major cytosol enzyme. The enzyme eluted at a potassium phosphate concentration of about

0.1 M. The active fractions were combined and after addition of 0.25 M sucrose, 1 mM EDTA and 1 mM DTT, the eluate was concentrated using an Amicon ultrafiltration cell with a PM10 membrane. The concentrated enzyme was stored in the deep freeze at -20°.

Purification of mitochondrial phosphoenolpyruvate carboxykinase

Mitochondrial extracts were prepared as described above (p.174) using 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT. The enzyme could be purified from extracts which had been frozen at -20° for up to one month, since there was negligible loss of activity during this time.

DEAE-Sephadex A-50 chromatography

Since the mitochondrial extract had a low protein concentration and a small volume, the ammonium sulphate fractionation and Sephadex G-100 steps carried out during purification of cytosol PEPCK were omitted. The mitochondrial extract (10 - 15 ml) was applied directly to a DEAE-Sephadex A-50 column as described for the cytosol enzyme (p.177). Elution was carried using 800 ml of a linear gradient of 0-0.375 M sodium chloride in 50 mM Tris-HCl, pH 8.0. The mitochondrial enzyme eluted as a single peak at a sodium chloride concentration of about 0.08 M. The active fractions were combined and the solution was brought to 70% saturation with solid ammonium sulphate. The protein precipitate was collected and dissolved in the minimum volume of 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT.

At this stage the enzyme was stable and could be stored at -20° for up to 2 months without loss of activity.

Protein did not precipitate from solution at ammonium sulphate concentrations lower than 60% saturation, hence an ammonium sulphate fractionation step was not feasible at this stage.

Sephadex G-200 chromatography

The concentrated enzyme (2 - 3 ml) was applied to a Sephadex G-200 column (0.64 cm^2 x 60 cm) which had been equilibrated with 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT. The enzyme was eluted with the same buffer at a flow rate of 0.12 ml/minute and 1 ml fractions were collected. The active fractions were combined and dialysed against 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT for 2 - 3 hours.

Hydroxylapatite chromatography

Hydroxylapatite chromatography was carried out as described above for cytosol PEPCK (p.178), using the dialysed Sephadex G-200 eluate (12 - 15 ml). The enzyme eluted at a potassium phosphate concentration of about 0.1 M. The active fractions were combined and after addition of 0.25 M sucrose, 1 mM EDTA and 1 mM DTT, the eluate was concentrated by dialysing against Carbowax for 2 - 3 hours. The concentrated enzyme was stored in the deep freeze at -20°.

Attempts to concentrate the hydroxylapatite eluate either by dialysis against saturated ammonium sulphate or by using an Amicon ultra-filtration cell were unsuccessful and resulted in loss of as much as 90% of the enzyme activity.

Brief account of other purification procedures

Attempts were made to separate mitochondrial and cytosol PEPCK using total homogenates and omitting the subcellular fractionation procedure. Homogenates were prepared in 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT, using a Waring blender. After centrifugation at 36,000 g, 4°, for 30 minutes using an MSE 18 High Speed refrigerated centrifuge, the supernatants were used. On one occasion the supernatant was applied directly to a

DEAE-Sephadex column as described on p.177 and elution was carried out using 1,600 ml of a linear gradient of 0 - 0.375 M sodium chloride in 50 mM Tris-HCl, pH 8.0. The enzyme eluted as a broad peak and there was no clear separation of the different forms. On another occasion, ammonium sulphate fractionation, Sephadex G-100 and DEAE-Sephadex chromatography were carried out as described on pp. 176 - 177, except that the enzyme was eluted from DEAE-Sephadex as described above. Again there was a broad peak and no clear separation. The DEAE-Sephadex eluate was then dialysed overnight against 50 mM Tris-HCl, pH 8.0 containing 1 mM DTT and re-applied to a DEAE-Sephadex column as before. Elution was carried out firstly with 300 ml of 0.15 M sodium chloride in 50 mM Tris-HCl, pH 8.0 and then with 300 ml of 0.35 M sodium chloride in 50 mM Tris-HCl, pH 8.0. Two peaks were obtained, the first one presumably corresponding to a mixture of mitochondrial PEPCK and the minor form found in the cytosol and the second one, eluting at the higher sodium chloride concentration, corresponding to the major cytosol form.

Initially, cellulose phosphate P11 chromatography was carried out using the method described by Chang and Lane (1966) for purification of pig liver mitochondrial PEPCK. The cellulose phosphate was equilibrated by washing successively with 0.1 N sodium hydroxide, water (until neutral), 0.1 N hydrochloric acid, water (until neutral), 500 mM potassium phosphate buffer, pH 7.0 and finally 5 mM potassium phosphate buffer, pH 7.0. After hydroxylapatite chromatography, the major cytosol enzyme was dialysed against 5 mM potassium phosphate buffer, pH 7.0 for 4 - 5 hours and then applied to the cellulose phosphate column which had been equilibrated with the same buffer. However, the enzyme, together with the majority of other proteins, was not adsorbed onto the column and no further purification was achieved during the procedure. This method was therefore not adopted.

When further purification of the mitochondrial and minor cytosol enzymes was attempted, after the hydroxylapatite step, using a cellulose phosphate P11 column as described for the cytosol enzyme (p.179), it was found that both forms were extremely unstable, even in the presence of 0.25 M sucrose in the buffer. Both forms eluted from the column similarly to the major cytosol enzyme but over 80% of the activity applied to the column was lost during the procedure.

MOLECULAR WEIGHT DETERMINATION

Approximate values for the molecular weights of PEPCK in the purified preparations were estimated by means of Sephadex G-200 chromatography. A column of dimensions 0.6 cm^2 x 75 cm was used and was equilibrated by washing with 50 mM Tris-HCl, pH 8.0 for 4 days prior to use.

The void volume of the column (V_o) was determined using Blue Dextran 2000. The column was then calibrated using commercial preparations of cytochrome c, haemoglobin, bovine plasma albumin, enolase (from rabbit muscle), aldolase (from rabbit muscle) and pyruvate kinase (from rabbit muscle).

The standards were applied individually to the column (5 - 8 mg protein in a volume of 0.5 ml) and elution was carried out using 50 mM Tris-HCl, pH 8.0 at a flow rate of 0.2 ml/minute. The eluates from the column were passed through an LKB Uvicord absorptiometer and in each case the elution volume (V_e) was determined from the recording of optical density at 254 nm. A calibration curve was constructed by plotting elution volume against log (molecular weight) (Andrews, 1964).

The purified PEPCK preparations (1 - 2 U enzyme activity in a volume of 0.5 ml) were then applied to the column, individually. Elution was carried out in the same way and enzyme activity was assayed using

the radioactive carboxylation method described previously (p.167). The molecular weights corresponding to the elution volumes obtained were read from the calibration curve.

Bovine plasma albumin, which had been chromatographed at an early stage during the experiment, was rechromatographed at the end of the experiment, in order to check that the elution properties of the column had not changed and that the value obtained for the elution volume was the same as the initial value.

KINETIC STUDIES

Before kinetic studies were carried out, the purified enzyme preparations were dialysed against the appropriate assay buffer containing 1 mM DTT, for at least 1 hour. The assay methods used and the changes in substrate and cofactor concentrations in individual experiments were as indicated in the Results. All assays were carried out in duplicate. Concentrations of substrate solutions were determined using the methods described previously (p.165). Oxaloacetate solutions were always freshly prepared and were assayed immediately before use as well as at the end of the experiment, to make sure that no decomposition had occurred.

Determination of Michaelis constants

Reciprocal velocities were plotted against reciprocal substrate concentrations (Lineweaver and Burk, 1934). When the plots were linear, the data were fitted to the equation below,

$$v = \frac{VA}{K + A}$$

where v is the observed velocity, V is the maximum velocity, K is the apparent Michaelis constant and A is the substrate concentration. The

computer programme of Cleland (1963) was used. This programme is based on a least-squares fit of the data points to the equation and gives values for the Michaelis constants and their standard errors. The weighted means of the Michaelis constants and their standard errors were obtained as described by Morrison and Uhr (1966).

RESULTS

The subcellular distribution of phosphoenolpyruvate carboxykinase (PEPCK) in monkey liver was studied and the enzyme was purified from the cytosol and mitochondrial fractions. The two major forms, one from the cytosol fraction and one from the mitochondria, were characterised. In addition, some of the properties of a minor form occurring in the cytosol were examined.

Subcellular distribution of PEPCK in monkey livers

The relative activities of PEPCK and the marker enzymes glutamate dehydrogenase and lactate dehydrogenase in the nuclear, mitochondrial and cytosol fractions of monkey livers are shown in Table I. A small proportion of glutamate dehydrogenase activity was detected in the cytosol fractions, indicating only slight leakage from the mitochondria. The mitochondria were only slightly contaminated by cytosol enzymes, as indicated by the lactate dehydrogenase activities. PEPCK was almost equally distributed between the mitochondrial and cytosol fractions with a small proportion in the nuclear fraction.

Purification of PEPCK from monkey livers

The cytosol and mitochondrial fractions of monkey liver were separated by means of differential centrifugation. The cytosol preparations used contained 7 - 10% of the total glutamate dehydrogenase activity recovered in the nuclear, mitochondrial and cytosol fractions, indicating that there had been only slight leakage from the mitochondria. The mitochondrial preparations used contained only 1 - 2% of the total lactate dehydrogenase activity recovered in the nuclear, mitochondrial and cytosol fractions, indicating that there was negligible contamination by cytosol enzymes.

TABLE I

SUBCELLULAR DISTRIBUTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN MONKEY LIVERS

Subcellular fractions were prepared by differential centrifugation using 5 g of liver. Glutamate dehydrogenase and lactate dehydrogenase were used as marker enzymes for the mitochondrial and cytosol fractions respectively. The results given are means (± S.E.M.) of those obtained on three occasions.

ENZYME	SPECIFIC ACTIVITY OF HOMOGENATE†† (mU/mg protein)	% OF RECOVERED ACTIVITY*			% RECOVERY‡†
		NUCLEI	MITOCHONDRIA	CYTOSOL	
Phosphoenolpyruvate† carboxykinase	106.8 ± 13.2	9.8 ± 2.3	47.7 ± 3.5	42.5 ± 5.7	80.1 ± 3.7
Glutamate dehydrogenase	117.2 ± 3.4	6.8 ± 1.6	83.9 ± 3.3	9.3 ± 2.3	93.8 ± 4.3
Lactate dehydrogenase	1186 ± 56	1.2 ± 0.6	3.9 ± 0.9	94.9 ± 1.2	94.1 ± 4.8

†PEPCK activity was determined using the radioactive carboxylation assay.

††Protein was determined using the method of Lowry *et al.* (1951).

*Recovered activity = sum of activities in nuclear, mitochondrial and cytosol fractions. Phosphoenolpyruvate carboxykinase activity was not detected in microsomal fractions.

‡†% Recovery = $\frac{100 \times \text{sum of activities in nuclear, mitochondrial and cytosol fractions}}{\text{total activity in homogenate}}$

Summaries of the procedures used during the purification of cytosol PEPCK, together with typical results obtained, are given in Tables II and III. The initial stages of purification involved ammonium sulphate fractionation (30 - 55%), Sephadex G-100 and DEAE-Sephadex chromatography. Typical elution profiles obtained during Sephadex G-100 and DEAE-Sephadex A-50 chromatography are shown in Fig. 1. The enzyme eluted from Sephadex G-100 as a single peak, but on DEAE-Sephadex, two peaks were obtained. The minor and major peaks eluted at sodium chloride concentrations of about 0.08 and 0.15 M, respectively. On three different occasions, the proportion of the minor form was found to be 12.3, 18.4 and 21.6% of the total activity recovered after DEAE-Sephadex chromatography.

The major cytosol form of PEPCK was purified further by means of hydroxylapatite and cellulose phosphate P11 chromatography and the elution profiles obtained are included in Fig. 1. The minor form occurring in the cytosol was also purified further by means of hydroxylapatite chromatography and the elution profile obtained was very similar to that shown for the major form (Fig. 1). Both forms eluted from hydroxylapatite at a potassium phosphate concentration of about 0.1 M.

A summary of the procedures used during the purification of mitochondrial PEPCK, together with the results obtained, is given in Table IV. The purification involved DEAE-Sephadex A-50, Sephadex G-200 and hydroxylapatite chromatography. Typical elution profiles obtained during these procedures are shown in Fig. 2. In each case the enzyme eluted as a single peak. The enzyme eluted from DEAE-Sephadex A-50 at a sodium chloride concentration of about 0.08 M and from hydroxylapatite at a potassium phosphate concentration of about 0.1 M.

Stability of the purified enzymes

An aliquot of each of the final concentrated enzyme preparations

TABLE II

PURIFICATION OF CYTOSOL PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM MONKEY LIVER

MAJOR FORM

PURIFICATION STAGE	VOLUME (ml)	TOTAL ACTIVITY* (U)	TOTAL PROTEIN† (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %	PURIFICATION FACTOR PER STEP	OVERALL PURIFICATION FACTOR
Crude extract	262	232	8845	0.026	100	1	1
Ammonium sulphate precipitate (30-55%)	30	206	4140	0.049	89	1.9	1.9
Sephadex G-100 eluate	198	152	2079	0.073	66	1.5	2.8
DEAE-Sephadex eluate	208	92.9	218	0.426	40	5.8	16
Ammonium sulphate precipitate (0-70%)	8.7	89.9	208	0.432	39	1	17
Hydroxylapatite eluate	55	72.1	63.5	1.14	31	2.6	44
Cellulose phosphate eluate	51	48.1	14.7	3.27	21	2.9	125
Final concentrated preparation	5.2	39.8	12	3.32	17	1	128

*Enzyme activity was determined using the radioactive carboxylation assay as described in Methods.

†Protein was determined using the method of Warburg and Christian (1942).

TABLE III

PURIFICATION OF CYTOSOL PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM MONKEY LIVER

MINOR FORM

	PURIFICATION STAGE	VOLUME (ml)	TOTAL ACTIVITY* (U)	TOTAL PROTEIN† (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %	PURIFICATION FACTOR PER STEP	OVERALL PURIFICATION FACTOR
TOTAL	Crude extract	262	232	8845	0.026	100	1	1
TOTAL	Ammonium sulphate precipitate (30-55%)	30	206	4140	0.049	89	1.9	1.9
TOTAL	Sephadex G-100 eluate	198	152	2079	0.073	66	1.5	2.8
MINOR FORM	DEAE-Sephadex eluate	224	14.5	140	0.104	6.2	1.4	4
MINOR FORM	Ammonium sulphate precipitate (0-70%)	8.8	12.9	135	0.096	5.5	0.9	3.7
MINOR FORM	Hydroxylapatite eluate	60	10.8	58.5	0.185	4.6	1.9	7.1
MINOR FORM	Final concentrated preparation	6.4	10.7	52.8	0.203	4.6	1.1	7.8

*Enzyme activity was determined using the radioactive carboxylation assay as described in Methods.

†Protein was determined using the method of Warburg and Christian (1942).

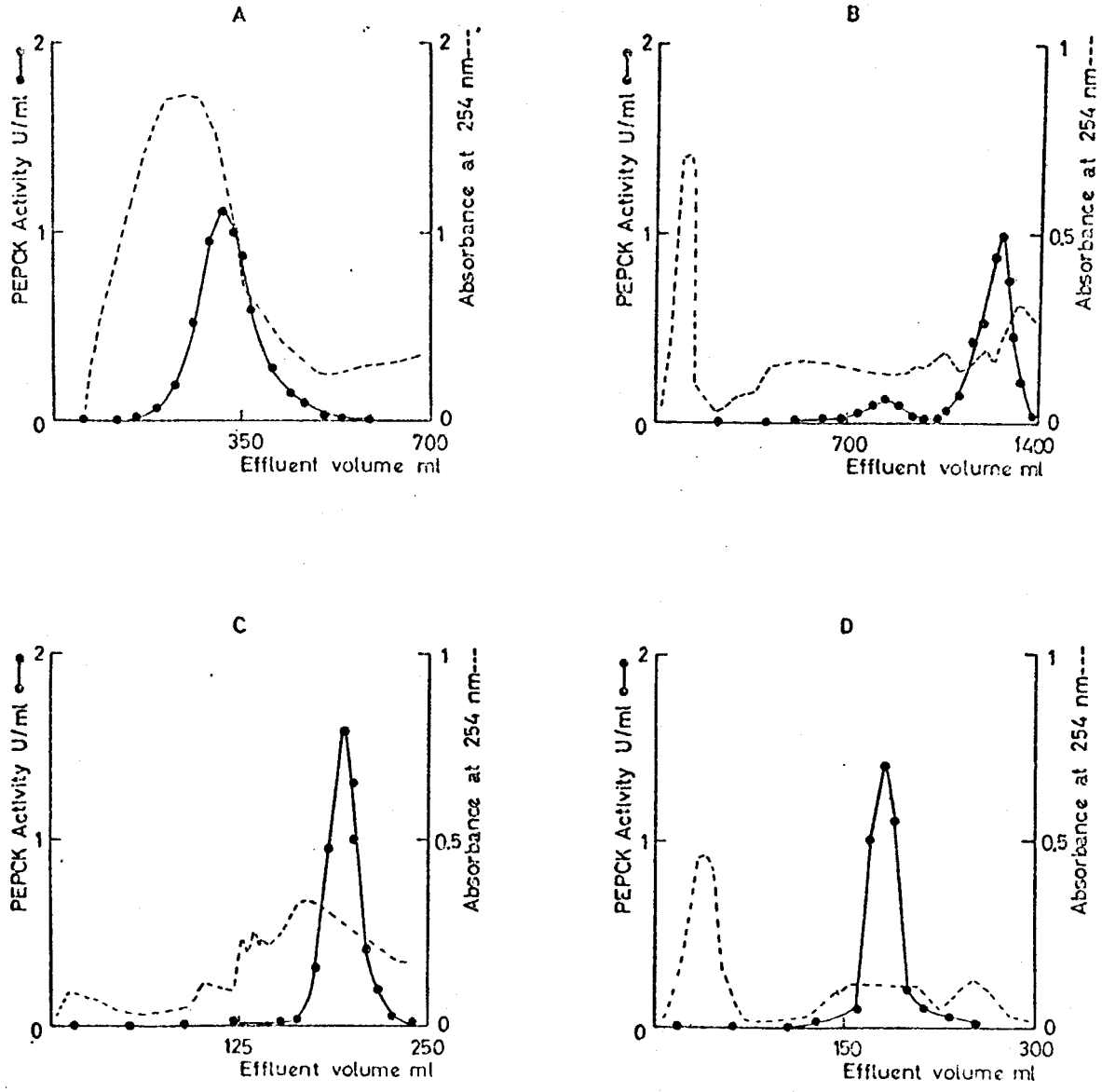


FIGURE 1. Elution profiles obtained during the purification of cytosol PEPCK from monkey liver. A. Sephadex G-100. B. DEAE-Sephadex A-50; the first gradient (0-0.15 M NaCl) was applied after 200 ml of effluent had been collected and the second gradient (0.15-0.375 M NaCl) after a further 800 ml had been collected. C. Hydroxylapatite; the gradient was applied after 50 ml of effluent had been collected. D. Cellulose phosphate P11; enzyme was eluted after 90 ml of effluent had been collected. Enzyme activity was determined using the radioactive carboxylation assay. Other details are given in Methods.

TABLE IV

PURIFICATION OF MITOCHONDRIAL PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM MONKEY LIVER

PURIFICATION STAGE	VOLUME (ml)	TOTAL ACTIVITY* (U)	TOTAL PROTEIN† (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %	PURIFICATION FACTOR PER STEP	OVERALL PURIFICATION FACTOR
Crude extract	14.5	171	1560	0.11	100	1	1
DEAE-Sephadex eluate	110	134	92.4	1.45	78	13.2	13
Ammonium sulphate precipitate (0-70%)	3	129	82.8	1.56	75	1.1	14
Sephadex G-200 eluate	15	117	38.1	3.07	68	1.9	28
Hydroxylapatite eluate	54	101	7.04	14.4	59	4.7	131
Final concentrated preparation	9.6	95.5	6.54	14.6	55	1	133

*Enzyme activity was determined using the radioactive carboxylation assay as described in Methods.

†Protein was determined using the method of Warburg and Christian (1942).

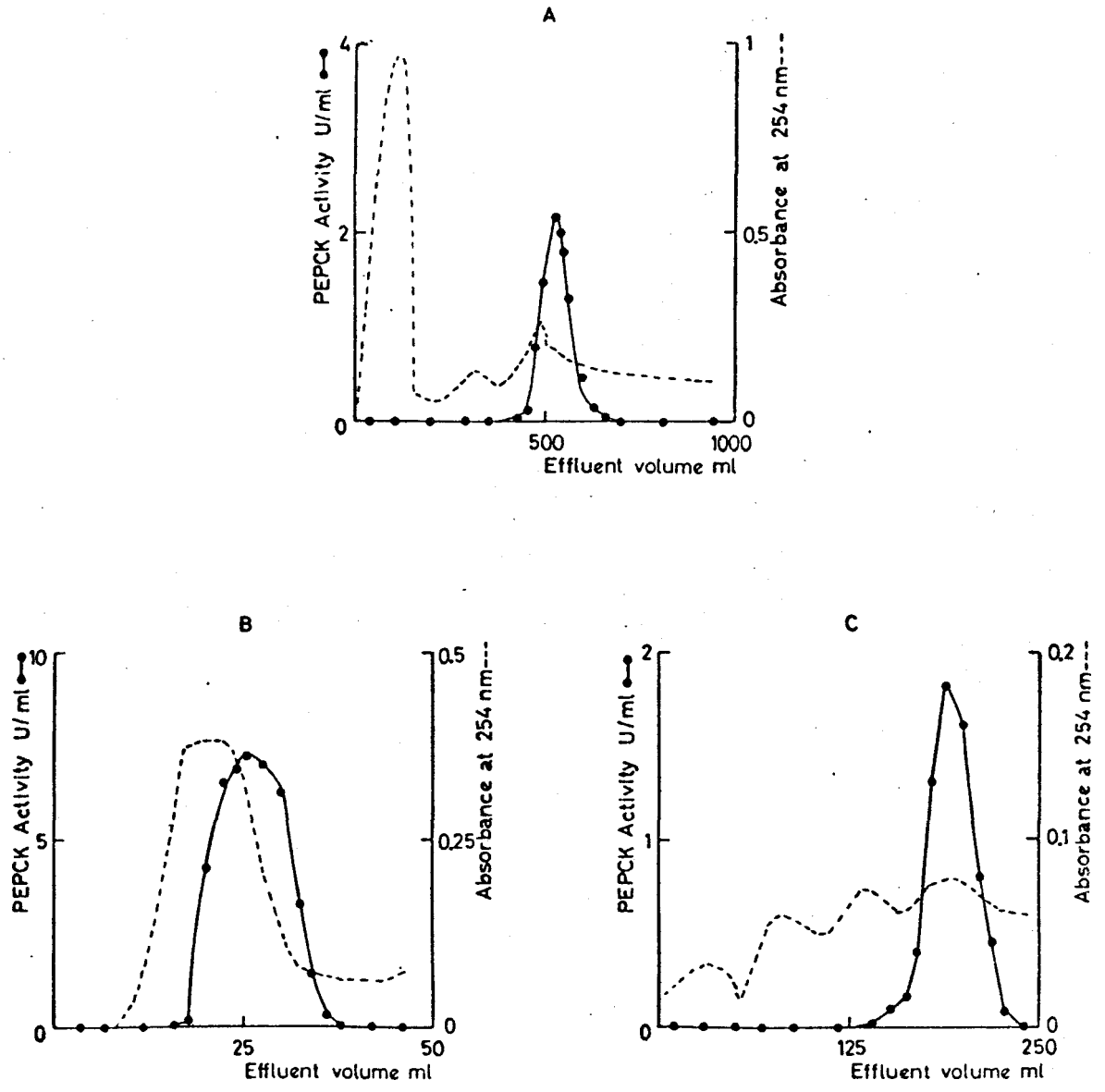


FIGURE 2. Elution profiles obtained during the purification of mitochondrial PEPCK from monkey liver. A. DEAE-Sephadex A-50; the gradient was applied after 200 ml of effluent had been collected. B. Sephadex G-200. C. Hydroxylapatite; the gradient was applied after 50 ml of effluent had been collected. Enzyme activity was determined using the radioactive carboxylation assay. Other details are given in Methods.

containing 0.25 M sucrose, 1 mM EDTA and 1 mM DTT was kept in the deep freeze at -20°. The activity of PEPCK in each aliquot was re-assayed after 1, 2 and 3 months and the results obtained are shown in Table V. There was only slight loss of activity during the three-month period.

The concentrated cytosol and mitochondrial preparations were dialysed against imidazole-HCl, pH 7.0 containing 1 mM DTT and then diluted with the same buffer so as to produce activity levels suitable for assay during kinetic studies. The diluted enzymes were then kept on ice at 0° for 4 hours and it was found that there was negligible loss of activity during this time.

Molecular weights of cytosol and mitochondrial PEPCK

For the standards used, pyruvate kinase (molecular weight 237,000), aldolase (molecular weight 158,000), enolase (molecular weight 84,760), bovine plasma albumin (molecular weight 67,000), haemoglobin (molecular weight 64,500) and cytochrome c (molecular weight 12,270), the respective elution volumes relative to the void volume of the Sephadex G-200 column (V_e/V_o) were 1.25, 1.53, 1.75, 1.89, 1.97 and 2.68. For both the major and minor cytosol forms and also for mitochondrial PEPCK, the value of this ratio was 1.95. The elution volumes obtained for cytosol and mitochondrial PEPCK corresponded in each case to molecular weights of approximately 66,000.

Kinetic studies

Substrates

The substrates PEP, IDP and GDP, used for the carboxylation reaction, were found to be 96 - 98% pure when assayed. The IDP and GDP preparations used did not contain ITP or GTP, respectively, which would

TABLE V

STABILITY OF PURIFIED PHOSPHOENOLPYRUVATE CARBOXYKINASE PREPARATIONS

The final concentrated enzyme preparations were stored at -20° in the presence of 0.25 M sucrose, 1 mM EDTA and 1 mM DTT.

STORAGE TIME	% INITIAL ACTIVITY*		
	CYTOSOL PEPCK		MITOCHONDRIAL PEPCK
	MAJOR FORM	MINOR FORM	
1 month	99.6	99.4	99.3
2 months	97.3	96.5	95.5
3 months	96.1	95.2	92.6

*Enzyme activity was determined using the radioactive carboxylation assay as described in Methods.

inhibit the enzyme (see p.196). The ITP and GTP preparations used for the decarboxylation reaction were found to be 97 - 99% pure when assayed and to be free from IDP and GDP, respectively. Some of the commercial ITP and GTP preparations, however, were found to be only 70 - 80% pure when assayed and to contain the respective nucleoside diphosphates which are inhibitors of the decarboxylation reaction (see p.197).

Oxaloacetate solutions of concentration 0.5 mM prepared in assay buffer (100 mM imidazole-HCl, pH 7.0) were found to be stable for up to 2 hours, but after 4 hours, 7% decomposition had occurred. Solutions of concentration 0.05 mM or lower were less stable, with 20 - 30% decomposition occurring within 1 hour. Oxaloacetate solutions were therefore prepared and assayed immediately before use.

Interfering enzymes

The activities of enzymes (expressed as percentages of the corresponding PEPCK activities), which would interfere during kinetic studies namely, pyruvate kinase, enolase, 'inosine diphosphatase', malate dehydrogenase and 'inosine triphosphatase', in PEPCK preparations at various stages of purification are shown in Table VI. There was significant pyruvate kinase contamination in cytosol preparations after DEAE-Sephadex A-50 chromatography, but the activity of this enzyme was considerably reduced after hydroxylapatite chromatography and was negligible in the final preparations. Pyruvate kinase activity was not detected in mitochondrial preparations.

Enolase, 'inosine diphosphatase' and 'inosine triphosphatase' activities were not detected in any of the preparations assayed.

There was significant malate dehydrogenase activity in cytosol and mitochondrial preparations after DEAE-Sephadex A-50 chromatography.

TABLE VI

INTERFERING ENZYMES IN PHOSPHOENOLPYRUVATE CARBOXYKINASE PREPARATIONS

ENZYME	PURIFICATION STAGE	ENZYME ACTIVITY EXPRESSED AS % PEPCK ACTIVITY†		
		CYTOSOL PEPCK		MITOCHONDRIAL PEPCK
		MAJOR FORM	MINOR FORM	
Pyruvate kinase	DEAE-Sephadex eluate	12.5	5.4	0*
	Hydroxylapatite eluate	0.62	0	0
	Cellulose phosphate eluate	0.15		
Enolase	DEAE-Sephadex eluate	0	0	0
	Hydroxylapatite eluate	0	0	0
	Cellulose phosphate eluate	0		
Inosine diphosphatase	Hydroxylapatite eluate	0	0	0
	Cellulose phosphate eluate	0		
Malate dehydrogenase	DEAE-Sephadex eluate	25.2	239	5.82
	Hydroxylapatite eluate	13.9	163	0.12
	Cellulose phosphate eluate	0		
Inosine triphosphatase	Hydroxylapatite eluate	0	0	0
	Cellulose phosphate eluate	0		

*O-Activity was not detected using 2-3 times the amount of enzyme required for PEPCK assay.

†Activities of interfering enzymes were determined as described in Methods. PEPCK activity was determined using the radioactive carboxylation assay.

After hydroxylapatite chromatography, the activity was only slightly reduced in the cytosol preparations, but was negligible in the final mitochondrial preparation. However, when the major cytosol enzyme was purified further by means of cellulose phosphate P11 chromatography, malate dehydrogenase activity was not detected.

Properties of the two major forms of PEPCK in monkey liver

The purified cytosol and mitochondrial preparations used had specific activities of 3.3 and 14.6 U/mg, respectively, and were not contaminated with enzymes which would interfere during the kinetic studies. Nucleoside phosphate specificity, pH optima, metal-ion activation, Michaelis constants for the various substrates and the effects of possible modulators were studied in the direction of oxaloacetate synthesis (carboxylation) as well as in the physiological direction of phosphoenolpyruvate synthesis (decarboxylation).

Nucleoside phosphate specificity

The relative activities of the enzymes with inosine, guanosine, adenosine, cytidine and uridine di- and triphosphates were determined. The results obtained are given in Table VII. Under the conditions used (given in Table VII), substrates and cofactors were present at saturating concentrations.

For the carboxylation reaction, cytosol PEPCK was more active with GDP than with IDP, whereas the mitochondrial form was equally active with both substrates. Both forms showed only slight activity with ADP and were inactive with CDP or UDP.

For the decarboxylation reaction, the cytosol and mitochondrial forms were both more active with ITP than with GTP or UTP and showed only slight activity with ATP. With CTP, cytosol PEPCK showed some activity

TABLE VII

NUCLEOSIDE PHOSPHATE SPECIFICITY OF MONKEY LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE

REACTION	SUBSTRATE	% OF ACTIVITY WITH IDP OR ITP	
		CYTOSOL PEPCK	MITOCHONDRIAL PEPCK
Carboxylation*	IDP	100	100
	GDP	158	99
	ADP	0.4	0.7
	CDP	0	0
	UDP	0	0
Decarboxylation†	ITP	100	100
	GTP	58	67
	ATP	4.8	4.5
	CTP	24	0
	UTP	43	53

*Enzyme activity was determined using the radioactive method. The assay mixture contained 100 mM imidazole-HCl, 2 mM $MnCl_2$, 1 mM GSH, 50 mM $NaH^{14}CO_3$, 2.5 mM NADH, 3 mM PEP, 2 U MDH enzyme and 1.25 mM IDP, GDP, ADP, CDP or UDP at pH 7.0.

†Enzyme activity was determined using the continuous spectrophotometric method. The assay mixture contained 100 mM imidazole-HCl, pH 7.0, 0.5 mM OAA, 5 mM $MnCl_2$, 2.9 mM ADP, 0.15 mM NADH, 1 mM GSH, 4 U PK, 4 U LDH, enzyme and 5 mM ITP, GTP, ATP, CTP or UTP.

but the mitochondrial form was inactive.

pH Optima

The pH optimum curves obtained for the carboxylation and decarboxylation reactions are shown in Fig. 3. Substrates and cofactors were present at saturating concentrations at pH 7.0 for both reactions (assay conditions are given in Fig. 3). Sodium acetate-acetic acid (pH range 3.7 - 5.6), sodium cacodylate-HCl (pH range 5.0 - 7.4), imidazole-HCl (pH range 6.2 - 7.8) and Tris-HCl (pH range 7.1 - 8.9) buffers were substituted at the same concentrations for the buffers normally used in the assay systems. Measurements of pH were carried out on reaction mixtures which were prepared at the same time and were identical to the assay mixtures. The pH readings were taken immediately after the mixtures had been prepared and at intervals over a period of 10 minutes, using a Radiometer pH meter with scale expander. No change in pH from that initially observed was noted in any instance.

The shapes of the pH curves obtained for the two enzymes differed slightly. In the carboxylation direction, the cytosol and mitochondrial forms had sharp pH optima at pH 6.8 and 7.0, respectively. In the decarboxylation direction, the activity of the cytosol enzyme in the alkaline region decreased less sharply than that of the mitochondrial form. Activities were optimal in the region pH 7.0 - 8.0 for the cytosol enzyme and in the region pH 7.0 - 7.2 for the mitochondrial enzyme.

Metal-ion specificity

In the direction of oxaloacetate synthesis, the effects of varying concentrations of manganese chloride, magnesium chloride and iron II chloride at saturating substrate concentrations were studied and the

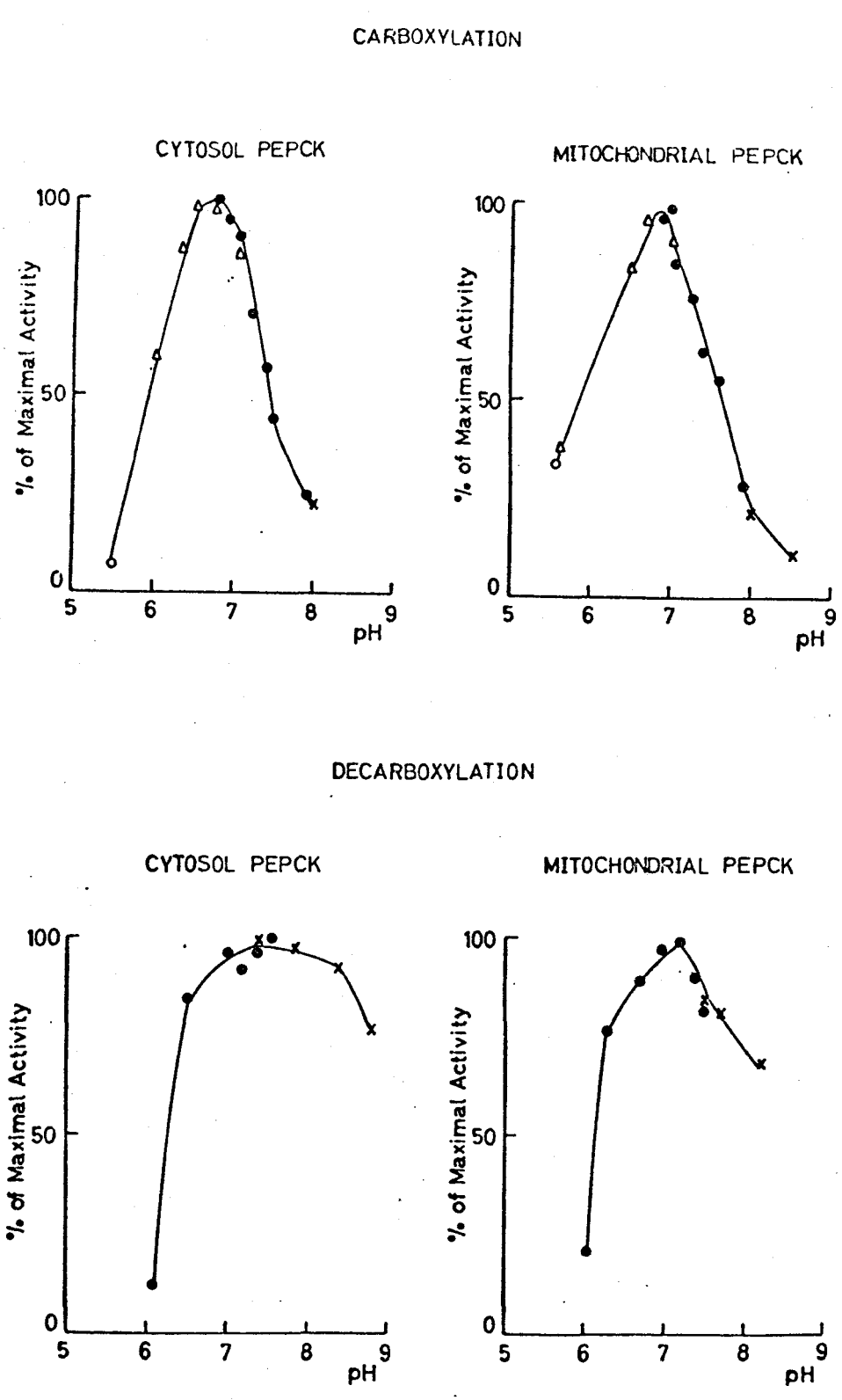


FIGURE 3. pH Optimum curves for cytosol and mitochondrial PEPCK from monkey liver. In the carboxylation direction, the radioactive assay was used; the reaction mixture contained buffer, 2 mM $MnCl_2$, 1 mM GSH, 50 mM $NaH^{14}CO_3$, 2.5 mM NADH, 3 mM PEP, 2 U MDH, 1.25 mM IDP and enzyme. In the decarboxylation direction, the Seubert-Huth assay was used; the reaction mixture contained buffer, 1 mM GSH, 5 mM $MnCl_2$, 1 mM OAA, 5 mM ITP and enzyme. The buffers used were sodium acetate-acetic acid (o), sodium cacodylate-HCl (Δ), imidazole-HCl (•) and Tris-HCl (x).

results obtained are shown in Fig. 4. The effects of varying concentrations of iron II sulphate were also examined and the results obtained were very similar to those shown for iron II chloride. During studies in which iron II salts were used, the reaction mixtures were prepared and kept at 0° until the assays were carried out, in order to prevent autoxidation of iron II to iron III. Iron II salts were used only up to concentrations of 10 mM, since at higher concentrations precipitation occurred in the reaction mixtures.

The cytosol and mitochondrial enzymes were both most active with manganese chloride and least active with magnesium chloride. However, when activities with magnesium chloride were expressed as percentages of the corresponding activities with manganese chloride (Fig. 4), it was apparent that cytosol PEPCK was relatively less active with magnesium than was the mitochondrial form. The activities of both forms with iron II chloride, relative to the activities with manganese chloride, were similar. Under the conditions used (see Fig. 4), maximal activities were obtained for both enzymes with 2 mM manganese chloride or 5 mM magnesium chloride or iron II chloride. There was only slight inhibition at metal-ion concentrations exceeding those required for maximal activity.

In the direction of phosphoenolpyruvate synthesis, the effects of varying concentrations of manganese chloride and magnesium chloride at saturating substrate concentrations were studied and the results obtained are shown in Fig. 5. The cytosol and mitochondrial enzymes were again both more active with manganese chloride than with magnesium chloride; the activity of the cytosol form with magnesium chloride, relative to that with manganese chloride, was lower than that of the mitochondrial form. For both enzymes, under the conditions used (see Fig. 5), maximal activities were obtained with 5 mM manganese chloride; at manganese chloride

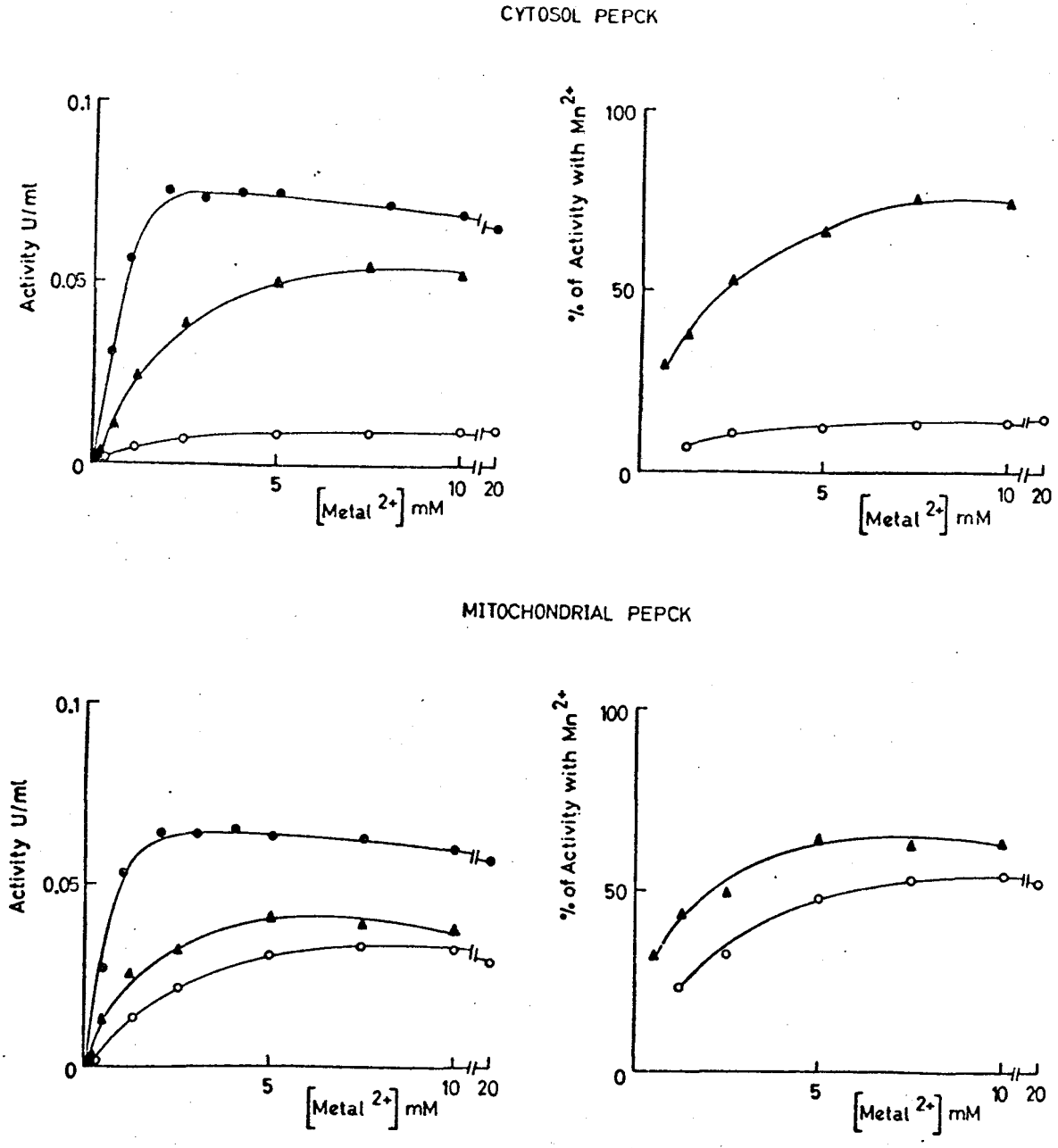


FIGURE 4. Effects of varying concentrations of metal ions on the activity of monkey liver cytosol and mitochondrial PEPCK in the carboxylation direction. The radioactive assay was used; the reaction mixture contained 100 mM imidazole-HCl, 1 mM GSH, 50 mM $NaH^{14}CO_3$, 2.5 mM NADH, 3 mM PEP, 2 U MDH, 1.25 mM IDP, enzyme and manganese chloride (●), iron II chloride (▲) or magnesium chloride (o) at the concentrations indicated. The final pH was 7.0.

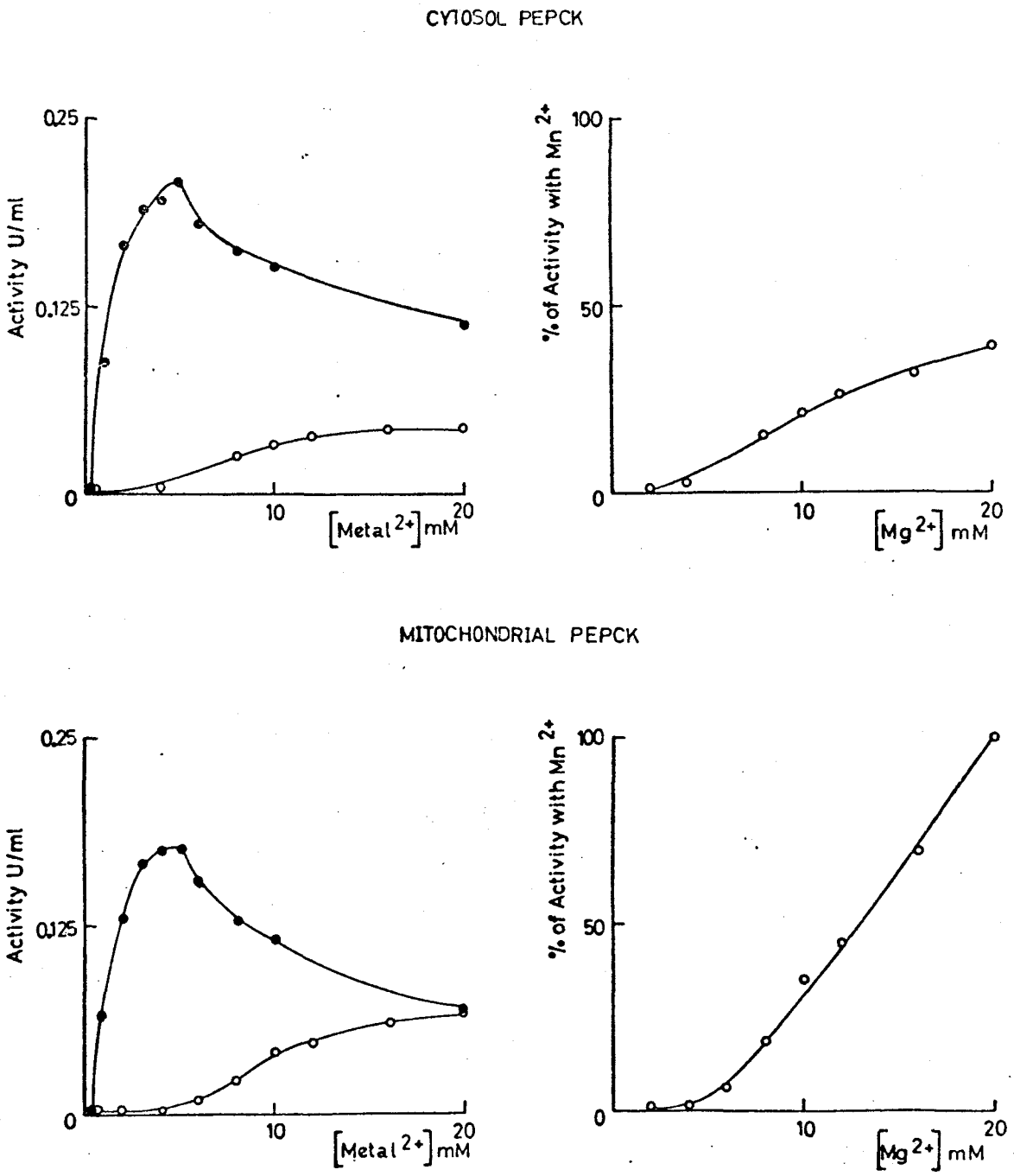


FIGURE 5. Effects of varying concentrations of metal ions on the activity of monkey liver cytosol and mitochondrial PEPCK in the decarboxylation direction. The Seubert-Huth assay was used; the reaction mixture contained 100 mM imidazole-HCl, pH 7.0, 1 mM GSH, 1 mM OAA, 5 mM ITP, enzyme and manganese chloride (●) or magnesium chloride (o) at the concentrations indicated.

concentrations exceeding 5 mM, the enzymes were inhibited. With magnesium chloride the activities increased up to 20 mM, which was the highest concentration tested.

Studies in which iron II chloride or sulphate replaced manganese or magnesium chloride in the reaction mixtures for the decarboxylation assay (the Seubert-Huth method was used) did not prove very satisfactory, since the solutions absorbed strongly at 340 nm. Reaction mixtures containing all reagents except oxaloacetate were colourless and did not absorb at 340 nm, but on addition of oxaloacetate the mixtures immediately turned reddish-brown and absorbed at 340 nm. The effect was not apparent in the absence of iron and was presumably due partly to formation of an oxaloacetate-iron complex and partly to oxidation of iron II to iron III, as a result of an increase in the rate of non-enzymic breakdown of oxaloacetate to pyruvate in the presence of metal ions. The intensity of the colour and the optical density at 340 nm increased with increasing iron II concentration and the effects were similar whether the assay mixtures were prepared either at 0° or at room temperature. When the pH of the assay buffer was decreased, the optical density at 340 nm decreased and was almost negligible at pH 6.2, but this pH was well below that required for optimal enzyme activity (see p.191). After the reactions had been stopped by addition of sodium borohydride, the optical density at 340 nm decreased and on addition of perchloric acid, the mixtures were almost colourless and the optical density at 340 nm was almost negligible (presumably as a result of decreasing the pH). When the mixtures were neutralised with potassium bicarbonate, the optical density at 340 nm again increased and continued to increase gradually with time even though the tubes were kept on ice. Phosphoenolpyruvate formed during the PEPCK reaction was determined at 340 nm and since the assay mixtures absorbed at this wavelength and absorbance increased with time, only very small aliquots (25 - 50 µl) could

be used. The results obtained using this system indicated that the activities of both PEPCK enzymes with iron II chloride or sulphate were similar to the activities with magnesium chloride and continued to increase up to 10 mM which was the highest concentration used. In view of the above, however, these results were not considered to be very reliable. The continuous spectrophotometric assay would not have been satisfactory either, since it involves measurement of the rate of disappearance of NADH at 340 nm.

Effects of manganese at varying nucleotide concentrations

The effects of varying manganese chloride concentrations at different concentrations of nucleotides are shown in Figs. 6 and 7. Concentrations of other substrates were saturating.

For the carboxylation reaction (Fig. 6), similar results were obtained for both enzymes. The amount of manganese chloride, relative to IDP or GDP, required for maximal activity decreased with increasing nucleotide concentration. Using 0.04, 1, 2 and 4 mM IDP, maximal activity was obtained with manganese chloride : IDP ratios of the order 30:1, 2:1, 2:1 and 1:1, respectively. The ratios manganese chloride : GDP required for maximal activity were of the order 100:1 and 2:1 for 0.005 and 1 mM GDP, respectively. In all cases, there was only slight inhibition at manganese chloride concentrations exceeding those required for maximal activity.

For the decarboxylation reaction (Fig. 7), using 0.2, 1, 2 or 4 mM ITP, it was found that the activities of both enzymes reached a maximum when the concentration of manganese chloride was equal to that of ITP. When the manganese chloride concentration exceeded that of ITP, both enzymes were somewhat inhibited. Similar effects were observed using

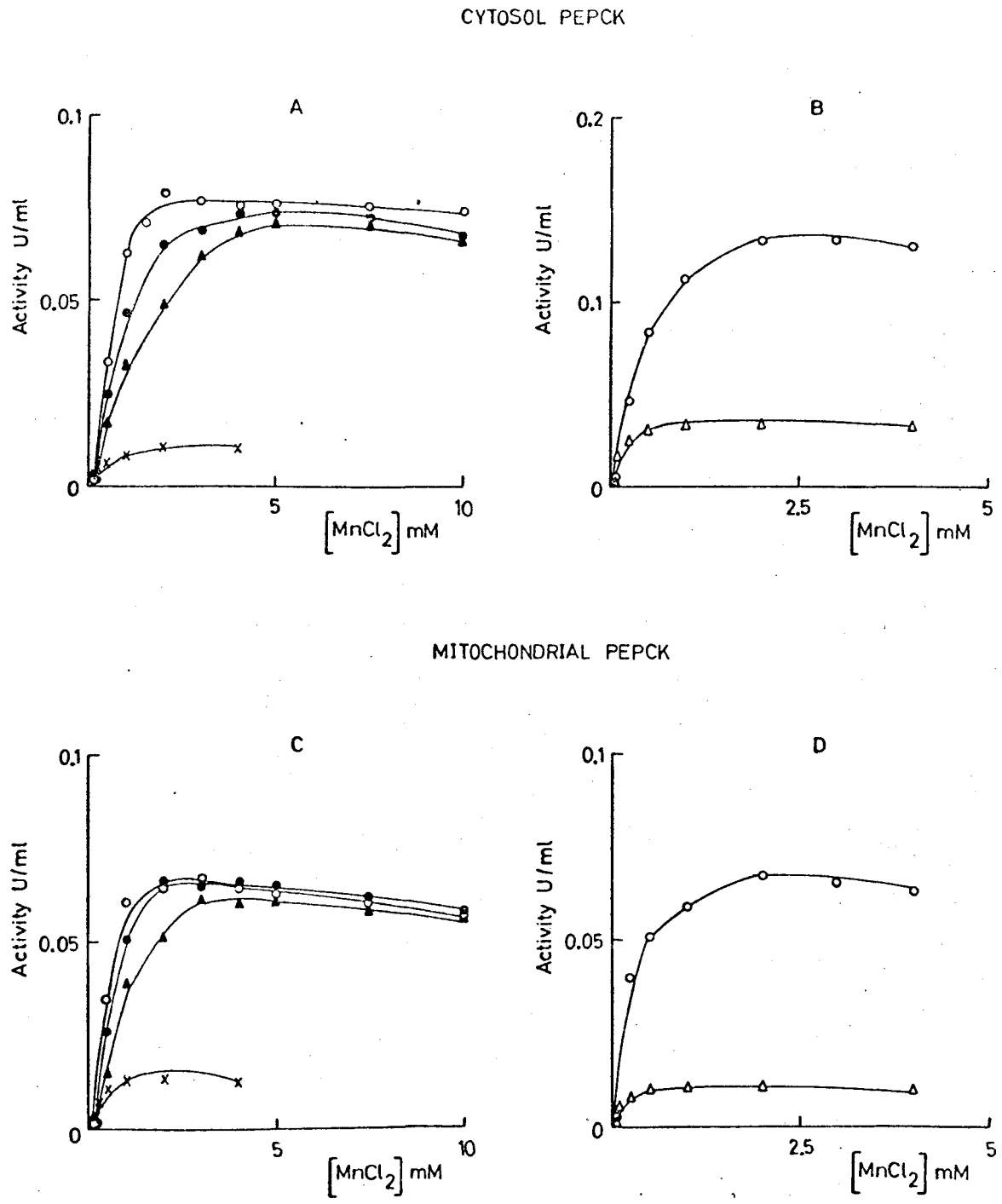


FIGURE 6. Effects of varying manganese chloride concentrations, at different concentrations of nucleoside diphosphate, on the activities of monkey liver cytosol and mitochondrial PEPCK in the carboxylation direction. The radioactive assay was used; the reaction mixture contained 100 mM imidazole-HCl, 1 mM GSH, 50 mM $NaH^{14}CO_3$, 2.5 mM NADH, 3 mM PEP, 2 U MDH, enzyme, manganese chloride at the concentrations indicated and 0.04 (x), 1 (o), 2 (•) or 4 (▲) mM IDP (Figs. A and C) or 0.005 (Δ) or 1 (o) mM GDP (Figs. B and D). The final pH was 7.0.

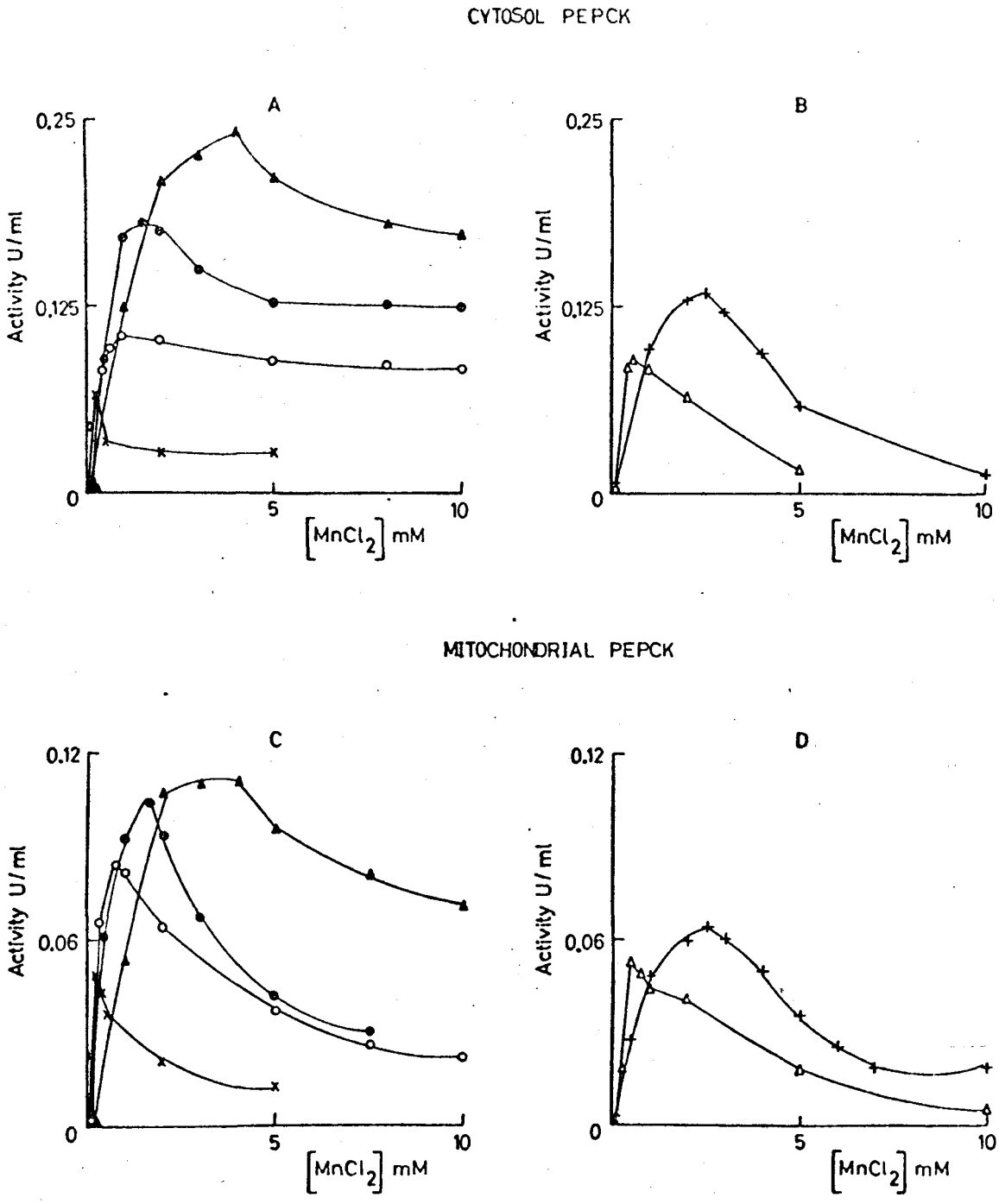


FIGURE 7. Effects of varying manganese chloride concentrations, at different concentrations of nucleoside triphosphate, on the activities of monkey liver cytosol and mitochondrial PEPCK in the decarboxylation direction. The Seubert-Huth assay was used; the reaction mixture contained 100 mM imidazole-HCl, pH 7.0, 1 mM GSH, 5 mM $MnCl_2$, 1 mM OAA, enzyme, manganese chloride at the concentrations indicated and 0.2 (x), 1 (o), 2 (•) or 4 (▲) mM ITP (Figs. A and C) or 0.5 (Δ) or 2.5 (+) mM GTP (Figs. B and D).

0.5 or 2 mM GTP.

Apparent Michaelis constants

The Lineweaver-Burk plots of reciprocal velocity *versus* reciprocal substrate concentration, obtained for cytosol and mitochondrial PEPCK, are shown in Figs. 8 and 9 and the mean values and standard deviations,obtained using the computer programme of Cleland (1963), are given in Table VIII. The Michaelis constants for each substrate were determined in the presence of saturating concentrations of the other substrates (assay conditions are given in Table VIII).

The Michaelis constants for phosphoenolpyruvate and sodium bicarbonate were determined in the presence of IDP and similar values were obtained for both the cytosol and mitochondrial enzymes. When determining the Michaelis constants for sodium bicarbonate, particular care was taken to ensure that all solutions were free from dissolved carbon dioxide and nitrogen was bubbled through the reaction mixtures prior to addition of enzyme. It is probable that the carbon species involved in the PEPCK-catalysed reaction is carbon dioxide and not bicarbonate. At equilibrium at pH 7.0, sodium bicarbonate solutions contain carbon dioxide and bicarbonate ion in the proportion 1:4, as determined from the Henderson-Hasselbalch equation using a value 6.4 for pK'_a (Jencks and Regenstein, 1973). The values given in Table VIII for the Michaelis constants of cytosol and mitochondrial PEPCK for sodium bicarbonate would therefore correspond to values 1.9 and 2.5 mM for carbon dioxide, respectively.

The Michaelis constants of cytosol PEPCK for IDP and GDP were slightly higher than those of mitochondrial PEPCK and for both forms the values for GDP were 10-fold lower than those for IDP.

TABLE VIII

APPARENT MICHAELIS CONSTANTS OF MONKEY LIVER

PHOSPHOENOLPYRUVATE CARBOXYKINASE

REACTION	SUBSTRATE	K_m (mM)	
		CYTOSOL PEPCK	MITOCHONDRIAL PEPCK
Carboxylation*	Phosphoenolpyruvate	0.14 ± 0.01	0.15 ± 0.01
	Sodium bicarbonate	9.7 ± 0.9	12.5 ± 1.1
	Inosine diphosphate††	0.29 ± 0.01	0.12 ± 0.01
	Guanosine diphosphate††	0.033± 0.001	0.014± 0.001
Decarboxylation†	Oxaloacetate	0.027± 0.002	0.022± 0.002
	Inosine triphosphate††	1.1 ± 0.1	0.32 ± 0.02
	Guanosine triphosphate	0.16 ± 0.01	0.13 ± 0.01

*Enzyme activity was determined using the continuous spectrophotometric method. The basic assay mixture contained 100 mM imidazole-HCl, 2 mM $MnCl_2$, 1 mM GSH, 0.15 mM NADH, 4 U MDH, 3 mM PEP, 1.25 mM IDP, 50 mM $NaHCO_3$ and enzyme at pH 7.0. Concentrations of PEP, $NaHCO_3$ or IDP (GDP) were varied within a suitable range.

†Enzyme activity was determined using the continuous spectrophotometric method. The basic assay mixture contained 100 mM imidazole-HCl, pH 7.0, 5 mM $MnCl_2$, 2.9 mM ADP, 0.15 mM NADH, 1 mM GSH, 4 U PK, 4 U LDH, 0.5 mM OAA, 5 mM ITP and enzyme. Concentrations of OAA or ITP (GTP) were varied within a suitable range. The ratio $MnCl_2$:ITP (GTP) was maintained at 1:1 throughout.

††The values given for the Michaelis constants for IDP, GDP and ITP are weighted means of three determinations.

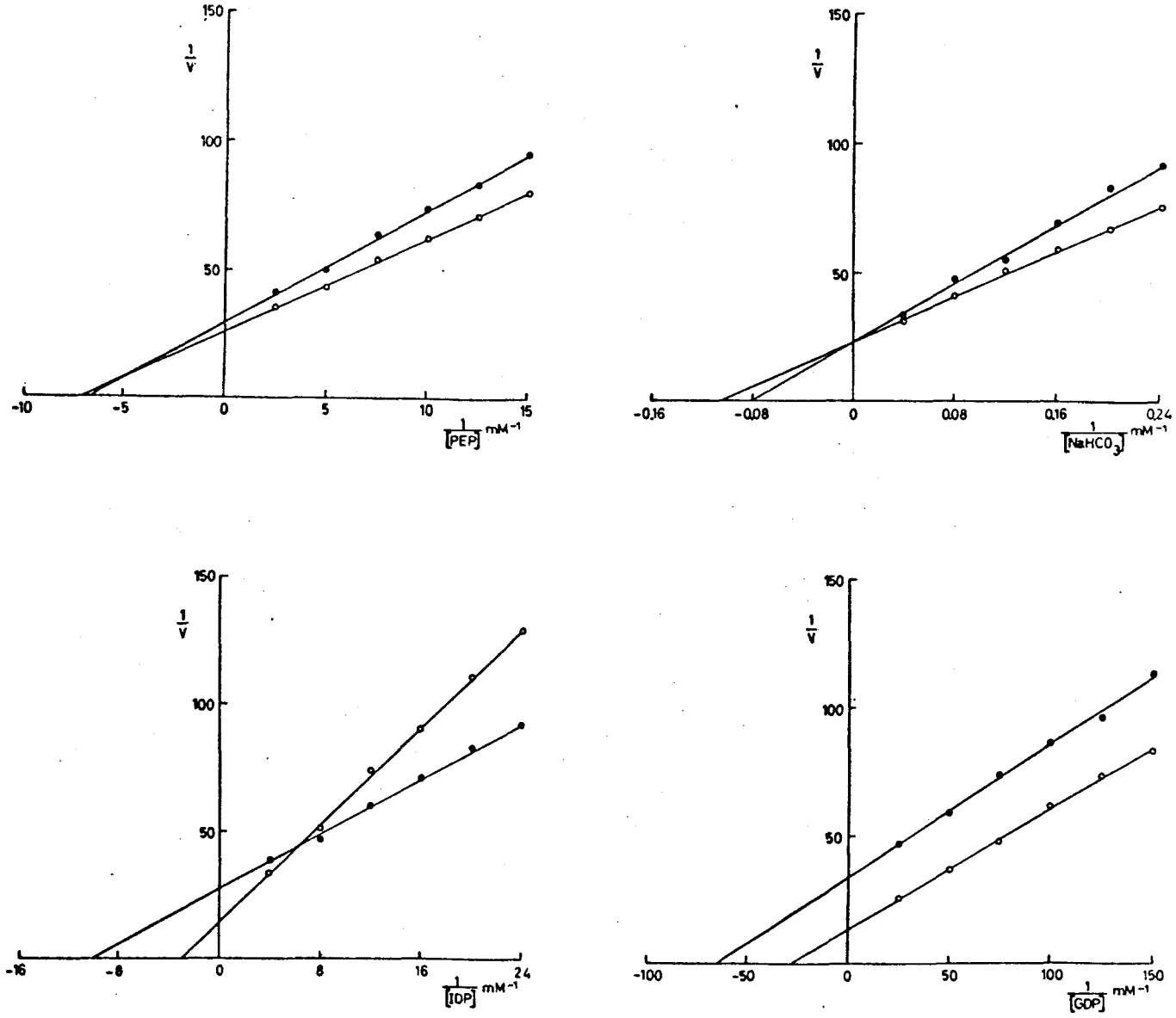


FIGURE 8. Lineweaver-Burk plots of reciprocal velocities against reciprocal substrate concentrations for monkey liver cytosol (o) and mitochondrial (●) PEPCK. The continuous spectrophotometric carboxylation assay was used and the basic reaction mixture was as described in Table VIII. Concentrations of each substrate were varied within the range indicated, in the presence of saturating concentrations of other substrates.

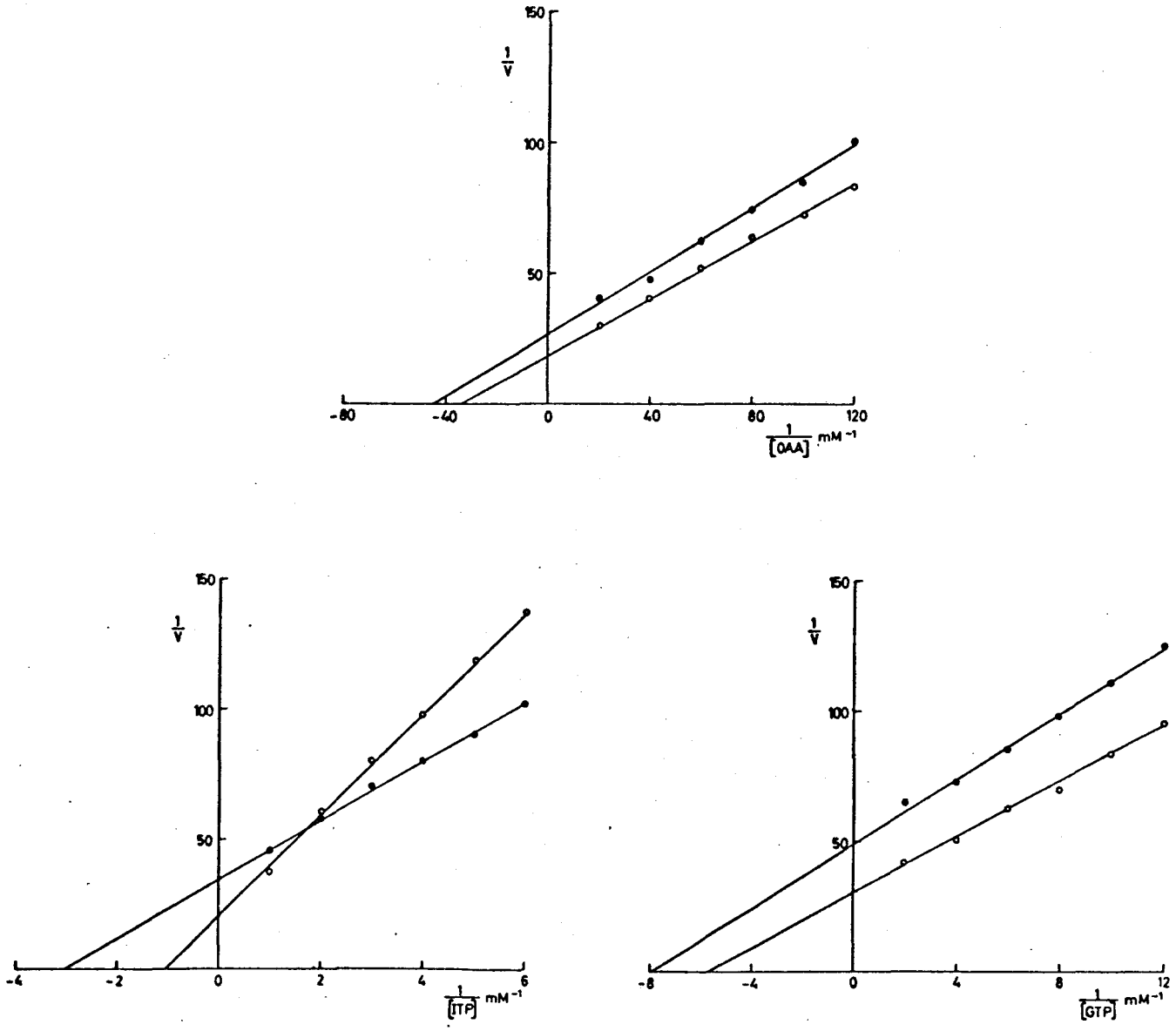


FIGURE 9. Lineweaver-Burk plots of reciprocal velocities against reciprocal substrate concentrations for monkey liver cytosol (o) and mitochondrial (●) PEPCK. The continuous spectrophotometric decarboxylation assay was used and the basic reaction mixture was as described in Table VIII. Concentrations of each substrate were varied within the range indicated, in the presence of saturating concentrations of other substrates.

The Michaelis constants for oxaloacetate were determined in the presence of ITP and similar values were obtained for both enzymes. No deviation from linearity was observed when oxaloacetate concentrations were varied over a wider range (3 - 100 μM).

The Michaelis constant of cytosol PEPCK for ITP was of the order 3-4-fold higher than that of mitochondrial PEPCK. The Michaelis constants obtained for GTP were similar for both enzymes. They were somewhat lower than those obtained for ITP, particularly so for cytosol PEPCK. Using the Seubert-Huth assay as described in Methods and varying ITP and manganese chloride concentrations within a suitable range, the value obtained for the Michaelis constant of mitochondrial PEPCK for ITP (0.37 mM) was similar to that given in Table VIII (0.32 mM) which was obtained using the continuous spectrophotometric method.

Effects of possible modulators

The effects of a number of compounds, including nucleotides, amino acids and citric acid cycle intermediates, on the activities of cytosol and mitochondrial PEPCK are shown in Tables IX and X. The required substances were dissolved in the appropriate assay buffer and where necessary the pH was adjusted to pH 7.0 before addition to the reaction mixtures.

In the carboxylation direction (Table IX), enzyme activity was determined in the presence of either manganese chloride or iron II sulphate. In the presence of manganese chloride, similar results were obtained for both enzymes and of the compounds tested only IMP, ITP, GTP, citrate, isocitrate and quinolinic acid had a significant inhibitory effect. When the manganese chloride concentration was increased to 6 mM in the presence of citrate or isocitrate, the activities were increased to 91 and 60%

TABLE IX

EFFECTS OF POSSIBLE MODULATORS ON MONKEY LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE

CARBOXYLATION REACTION

COMPOUND	CONCENTRATION (mM)	% OF THE ACTIVITY† IN THE ABSENCE OF THE COMPOUND TESTED			
		Mn^{2+} PRESENT		Fe^{2+} PRESENT	
		CYTOSOL PEPCK	MITOCHONDRIAL PEPCK	CYTOSOL PEPCK	MITOCHONDRIAL PEPCK
IMP	5	71.6	81.6	65	81.5
	50	19.4	30.3	18.7	17.1
AMP	50	72.6	81	69.2	74.5
ITP	5	4.7	13.5	91.5	98.8
GTP	5	7.9	15.9	63.7	82.4
L(+)-Alanine	100	98	97.2	113.9	117.7
L(+)-Arginine	100	80.8	86.1	86.7	105.8
L(+)-Homoarginine	50	75.9	89.9	66.2	95.2
L(+)-Cysteine	10	119	109	120	114.4
L(+)-Leucine	50	85	83.2	81.8	74.3
L(-)-Phenylalanine	50	79.6	84.8	72.7	73.4
L(-)-Histidine	20	90	85.2	89.1	91.6
L(-)-Tryptophan	10	100	99.4	91.5	38.2
Citrate	10	51.1	66	83.7	85.1
Isocitrate	50	36.3	40.2	47.1	46.5
α-Ketoglutarate	50	77.4	69.2	61.2	63.4
Succinate	25	84.2	78.8	98	93.2
Malate	50	78.2	79.1	78.4	76.4
Sodium fluoride	10	107	104	98.1	95.6
Hydrazine sulphate	10	99.5	103	90.3	91.6
	25	97.8	101		
	50	89.2	93.2		
Quinolinic acid	5	37.2	36.8	88.6	99.7

†Enzyme activity was determined using the radioactive assay. The assay mixture contained 100 mM imidazole-HCl, 1 mM GSH, 1.25 mM IDP, 3 mM PEP, 50 mM $NaH^{14}CO_3$, 2.5 mM NADH, 2 U MDH, enzyme and 2 mM $MnCl_2$ or 5 mM $FeSO_4$ at pH 7.0. The different compounds were added at the concentrations indicated.

TABLE X

EFFECTS OF POSSIBLE MODULATORS ON MONKEY LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE DECARBOXYLATION REACTION

COMPOUND	CONCENTRATION (mM)	% OF THE ACTIVITY† IN THE ABSENCE OF COMPOUND TESTED	
		CYTOSOL PEPCK	MITOCHONDRIAL PEPCK
IMP	5	83.4	100
AMP	50	83.4	62.9
IDP	5	54.2	88.5
GDP	5	5.5	28.6
L(+)-Alanine	100	99	92.3
L(+)-Arginine	100	87.4	92.4
L(+)-Homoarginine	50	81.5	87.6
L(+)-Cysteine	10	98.2	99.3
L(+)-Leucine	50	99.3	98.2
L(-)-Phenylalanine	50	81.4	83.1
L(-)-Histidine	20	90.6	91.4
L(-)-Tryptophan	10	86.2	100
Citrate	10	52.3	68.2
Isocitrate	50	40.1	66.5
α-Ketoglutarate	50	84.3	86.9
Succinate	25	90.4	93.1
Malate	50	89.8	90.7
Sodium fluoride	10	90.3	86.7
Hydrazine sulphate	10	65.5	91.6
Quinolinic acid	5	0	35.6

†Enzyme activity was determined using the continuous spectrophotometric method. The assay mixture contained 100 mM imidazole-HCl, pH 7.0, 1 mM GSH, 0.5 mM OAA, 5 mM ITP, 5 mM $MnCl_2$, 2.9 mM ADP, 0.15 mM NADH, 4 U PK, 4 U LDH and enzyme. The different compounds were added at the concentrations indicated.

of the control activities, respectively. Increasing the manganese chloride concentration to 10 mM did not affect the activities with quinolinic acid. AMP, the amino acids tested, α-ketoglutarate, succinate and malate were used at relatively high concentrations and did not affect the enzyme activities significantly. Sodium fluoride and hydrazine sulphate also had little effect. In many cases, the results obtained using iron II sulphate were similar to those obtained using manganese chloride. However, ITP, GTP, citrate and quinolinic acid, which were inhibitory in the presence of manganese chloride, were much less effective in the presence of iron II sulphate. The activity of the cytosol enzyme was virtually unaffected by tryptophan but the mitochondrial enzyme was somewhat inhibited when iron II sulphate was used.

In the decarboxylation direction (Table X), GDP, citrate, isocitrate and quinolinic acid inhibited both enzymes, but the cytosol form was affected to a slightly greater extent that the mitochondrial form. When 10 mM manganese chloride was used, the activities of the cytosol and mitochondrial forms in the presence of citrate increased to 89 and 94% of the control activities, respectively. Increasing the manganese chloride concentration to 10 mM did not affect the activities with quinolinic acid. IDP and hydrazine sulphate also inhibited cytosol PEPCK to some extent but had little effect on the mitochondrial form. The other compounds tested, IMP, AMP, amino acids, α-ketoglutarate, succinate, malate and sodium fluoride, many of which were used at relatively high concentrations, had no significant effect on the activities of either of the enzymes.

Properties of the minor form of PEPCK occurring in the cytosol

Some of the kinetic properties of the minor form of PEPCK isolated

from the cytosol were studied under the same conditions used during the corresponding experiments involving the two major enzymes. Although differences were observed between the major cytosol and mitochondrial forms, there were no apparent differences between the minor cytosol and the mitochondrial forms.

In the carboxylation direction, the minor cytosol form was equally active with IDP or GDP and showed only slight activity (0.8%) with ADP. In the decarboxylation direction, the activity with GTP was 52.6% of the activity with ITP. With ATP the activity was 5.2% of that with ITP (cf. Table VII).

The pH optimum curves obtained for the minor cytosol form were similar to those shown for mitochondrial PEPCK (Fig. 3), with optimal activities in the regions pH 7.0 for the carboxylation reaction and pH 7.0 - 7.2 for the decarboxylation reaction.

For the carboxylation reaction, the relative activities of the minor cytosol form with varying concentrations of manganese chloride, magnesium chloride and iron II chloride were similar to those shown for the mitochondrial enzyme (Fig. 4). The enzyme was most active with manganese chloride and least active with magnesium chloride, but as was also the case for the mitochondrial form, the activity with magnesium chloride was only slightly lower than that with iron II chloride. The minor cytosol enzyme reached maximal activity with 2 mM manganese chloride when 1.25 mM IDP was used and with 1 mM manganese chloride when 0.5 mM IDP was used.

For the decarboxylation reaction, the minor cytosol form was again more active with manganese chloride than with magnesium chloride and the relative activities at varying metal-ion concentrations were similar to those shown for mitochondrial PEPCK (Fig. 5). In the presence of 5 mM ITP,

the activity reached a maximum with 5 mM manganese chloride and then decreased as the manganese chloride concentration was increased further up to 20 mM. With magnesium chloride, in the presence of 5 mM ITP, the activity increased gradually reaching a maximum at 20 mM, which was the highest magnesium chloride concentration used.

The values obtained for the apparent Michaelis constants of the minor form were 0.15 ± 0.01, 12.9 ± 1.2, 0.11 ± 0.01 and 0.016 ± 0.002 mM for phosphoenolpyruvate, sodium bicarbonate, IDP and GDP, respectively (cf. Table VIII). Since the preparation was contaminated with malate dehydrogenase, Michaelis constants were not determined in the direction of phosphoenolpyruvate synthesis.

DISCUSSION

The key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), has been studied extensively in non-primate species (for a review see Utter and Kolenbrander, 1972) but has not previously been isolated from any primate tissue and characterised. In this work, PEPCK was purified from monkey liver and some of the catalytic properties of the cytosol and mitochondrial forms were studied.

In monkey livers, PEPCK was found to occur in almost equal amounts in the mitochondrial and cytosol fractions. This has also been shown to be the case in guinea pig, pig, cat, sheep, bovine and human livers (Nordlie and Lardy, 1963; Ballard *et al.*, 1968; Wieland *et al.*, 1968; Brech *et al.*, 1970; Söling *et al.*, 1970; Swiatek *et al.*, 1970; Garber and Hanson, 1971a; Smith and Osborne-White, 1971; Hanson and Garber, 1972). In rat, mouse and hamster livers, PEPCK is found mainly in the cytosol (Nordlie and Lardy, 1963; Ballard and Hanson, 1967) and in rabbit and avian livers, the enzyme is essentially mitochondrial (Utter, 1959; Nordlie and Lardy, 1963; Felicioli *et al.*, 1967; Gevers 1967; Johnson *et al.*, 1973).

The distribution of PEPCK in the liver cell has an important bearing on the mechanism and control of gluconeogenesis. In the rat, the proposed mechanism involves translocation of oxaloacetate across the mitochondrial membrane via the intermediates malate or aspartate prior to formation of phosphoenolpyruvate in the cytosol (Lardy *et al.*, 1965; Krebs *et al.*, 1967). In species in which PEPCK is present in the mitochondria, however, oxaloacetate can be converted directly to phosphoenolpyruvate (Arinze *et al.*, 1973; Hanson, 1974). The mitochondrial and cytosol forms of PEPCK appear to be subject to different control

mechanisms. In the livers of most mammalian species so far examined, only the cytosol enzyme is regulated by dietary or hormonal alterations (Nordlie *et al.*, 1965b; Gevers, 1967; Johnson *et al.*, 1970, 1973; Söling *et al.*, 1970; Garber and Hanson, 1971a, 1971b; Huibregtse *et al.*, 1976). The mitochondrial formation of phosphoenolpyruvate, in guinea pig and rabbit at least, appears to be controlled by the relative levels of NADH and NAD^+ (Garber and Hanson, 1971a, 1971b; Jomain-Baum and Hanson, 1973; Arinze *et al.*, 1973).

Two major forms of PEPCK were purified from monkey liver, one from the cytosol fraction and one from the mitochondria. For both enzymes the purification procedures used included DEAE-Sephadex A-50 and hydroxylapatite chromatography. The cytosol enzyme was eluted from DEAE-Sephadex at a higher sodium chloride concentration (0.15 M) than the mitochondrial enzyme (0.08 M), indicating a charge difference between the two forms. Both forms behaved similarly on hydroxylapatite chromatography and were eluted at a potassium phosphate concentration of approximately 0.1 M. Under conditions similar to those used in this study, Ballard and Hanson (1969) and Philippidis *et al.*, (1972), reported that rat liver cytosol PEPCK was eluted from DEAE-cellulose at a sodium chloride concentration of about 0.1 M and from hydroxylapatite at a potassium phosphate concentration of about 0.1 M.

The minor form of PEPCK which was isolated from the cytosol fraction of monkey liver could be either a separate enzyme, a form interconvertible with the major form, or it could be the mitochondrial enzyme which was present in the cytosol as a result of mitochondrial leakage during the subcellular fractionation procedure. The minor form was separated from the major cytosol form by means of DEAE-Sephadex A-50 chromatography and was eluted at a sodium chloride concentration similar

to that required for elution of the mitochondrial enzyme. Although often slightly higher than might have been expected as a result of mitochondrial leakage (as indicated by the amount of glutamate dehydrogenase activity in the cytosol fraction), the proportion of the minor form in the cytosol was relatively low. When some of the kinetic properties of the minor cytosol form of PEPCK were compared with those of the mitochondrial enzyme no differences were apparent. When assayed either in the direction of phosphoenolpyruvate synthesis or in the direction of oxaloacetate synthesis, the two forms were found to be similar with respect to nucleoside phosphate specificity, pH optima and metal-ion activation. Both forms had similar Michaelis constants for phosphoenolpyruvate, sodium bicarbonate, IDP and GDP. It therefore seems most likely that the minor form found in the cytosol was actually the mitochondrial enzyme and that there was in fact only one form of cytosol PEPCK in monkey liver. Further reference to cytosol PEPCK will therefore apply only to the major form.

In the livers of other mammalian species studied, rat, guinea pig, pig and sheep, no more than a single form of PEPCK has been reported in either the cytosol or mitochondrial fractions (Holten and Nordlie, 1965; Chang and Lane, 1966; Ballard and Hanson, 1969; Ballard, 1970). Additional forms of PEPCK have, however, been observed in the cytosol and mitochondrial fractions of chicken embryo and young chicken livers, as well as in the livers of adult chickens forced to exercise an active gluconeogenesis (Jo *et al.*, 1974).

The cytosol and mitochondrial forms of PEPCK from monkey liver had similar molecular weights, which were of the order 66,000. These were similar to the values 68,000, 71,100, 73,300 and 74,500 reported for the enzymes from chicken liver mitochondria (Utter and Kolenbrander, 1972),

sheep kidney mitochondria (Barns and Keech, 1972), pig liver mitochondria (Chang and Lane, 1966) and rat liver cytosol (Ballard and Hanson, 1969), respectively.

Some of the catalytic properties of the purified cytosol and mitochondrial enzymes from monkey liver were examined. The PEPCK-catalysed reaction is reversible (Utter and Kurahashi (1954b) have reported a value 2.69 at 30° for the equilibrium constant of the reaction as written on p.167) and studies were carried out both in the direction of oxaloacetate synthesis as well as in the physiological direction of phosphoenolpyruvate synthesis.

The results obtained showed that, although the cytosol and mitochondrial enzymes were similar in many respects, there were some differences between them. There were differences between the relative activities of the two enzymes with GDP in the carboxylation direction and with CTP in the decarboxylation direction. For the carboxylation reaction, the pH optima were similar for both enzymes, whereas for the decarboxylation reaction, the cytosol form had a broader pH optimum than the mitochondrial enzyme. When assayed in either direction, both enzymes were more active with manganese than with magnesium, although the activity with magnesium relative to that with manganese was lower for the cytosol enzyme than for the mitochondrial enzyme. The enzymes had similar Michaelis constants for phosphoenolpyruvate, sodium bicarbonate, oxaloacetate and GTP. The Michaelis constants of the cytosol enzyme for IDP, GDP and ITP were, however, somewhat higher than those of the mitochondrial enzyme. Studies of the effects of a number of possible modulators on enzyme activity showed that although the two forms were similar in many cases, there were differences with respect to inhibition by IDP, GDP, tryptophan, hydrazine sulphate and quinolinic acid.

In an earlier study, Holten and Nordlie (1965) compared the properties of PEPCK obtained from the cytosol and mitochondrial fractions of guinea pig liver. They found that the enzymes had similar pH optima in both directions and similar Michaelis constants for phosphoenolpyruvate, IDP, oxaloacetate, ITP and GTP. In the direction of oxaloacetate synthesis, both guinea pig liver enzymes showed an absolute specificity for manganese rather than magnesium, but in the direction of phosphoenolpyruvate synthesis the mitochondrial enzyme was more active with magnesium than manganese at pH 8.0, whereas the cytosol form was more active with manganese than with magnesium under all conditions tested. When assayed in the decarboxylation direction, the guinea pig liver mitochondrial enzyme was inhibited by AMP, whereas the cytosol form was not.

Ballard and Hanson (1967) found that the enzymes of adult rat liver cytosol and foetal rat liver mitochondria were similar with respect to relative activities with nucleoside diphosphates, pH optima in the carboxylation direction and Michaelis constants for phosphoenolpyruvate. Ballard (1970) showed that in the presence of manganese, the cytosol enzyme from sheep liver had a higher Michaelis constant for oxaloacetate than the mitochondrial enzyme and that the two forms differed with respect to inhibition by malate.

In view of the fact that they observed only slight differences between the cytosol and mitochondrial forms of PEPCK in guinea pig liver, Holten and Nordlie (1965) suggested that the two forms were variants of the same enzyme protein. Ballard and Hanson (1969), however, produced evidence that the cytosol and mitochondrial forms of PEPCK from rat liver were immunologically distinct proteins, despite their uniform kinetic properties. Even though the monkey liver enzymes were similar in certain

respects, the differences between the two forms, reported here, could suggest that they are distinct proteins.

It is possible that differences between the cytosol and mitochondrial forms of PEPCK from monkey liver may be of functional significance. At present, however, it is not clear what effects the slight differences in nucleoside phosphate specificity, pH optima and metal-ion activation and the higher Michaelis constants of the cytosol enzyme for IDP and ITP could have *in vivo*. Although interesting from a kinetic point of view, the higher Michaelis constant of cytosol PEPCK for GDP (0.033 mM), as compared with the value for the mitochondrial enzyme (0.014 mM), is unlikely to be of physiological importance, since the intracellular concentration of GDP (in rat liver) is in the 0.1 - 0.2 mM range (Clifford *et al.*, 1972), indicating that both enzymes would be fully saturated with respect to this nucleotide. The different effects of IDP, GDP, tryptophan, hydrazine sulphate and quinolinic acid on the cytosol and mitochondrial enzymes may reflect differences in the regulatory properties of the two forms.

The monkey liver enzymes were active with inosine or guanine nucleotides whereas with adenine nucleotides the activity was negligible, as is usually the case for PEPCK from higher species (Utter and Kolenbrander, 1972). When assayed in the direction of oxaloacetate synthesis, cytosol PEPCK from monkey liver was found to be more active with GDP than with IDP. The mitochondrial form was, however, equally active with both these substrates, which is consistent with the observations of Chang and Lane (1966) for pig liver mitochondrial PEPCK and Ballard and Hanson (1967) for the enzymes from adult rat liver cytosol and foetal rat liver mitochondria. The monkey liver enzymes showed negligible activity with UDP or CDP, as is also the case for adult rat liver cytosol and foetal

rat liver mitochondrial PEPCK (Ballard and Hanson, 1967). When assayed in the direction of phosphoenolpyruvate synthesis, the monkey liver enzymes were both found to be active with UTP as well as with ITP or GTP. The cytosol enzyme showed some activity with CTP whereas the mitochondrial form was inactive with this substrate. Both enzymes were less active with GTP or UTP than with ITP. Nordlie and Lardy (1963) showed that PEPCK from guinea pig liver mitochondria was less active with GTP than with ITP, whereas Chang and Lane (1966) reported that the enzyme from pig liver mitochondria was slightly more active with GTP than with ITP. Chang and Lane (1966) also found that the mitochondrial enzyme from pig liver was inactive with CTP but showed some activity with UTP.

For the carboxylation reaction, the monkey liver enzymes had sharp pH optima and the activity decreased rapidly with increasing pH in the alkaline region, as has also been reported for PEPCK from other species such as the pig, guinea pig and chicken (Utter *et al.*, 1954; Holten and Nordlie, 1965; Chang *et al.*, 1966; Utter and Kolenbrander, 1972). The one-carbon species involved in the PEPCK-catalysed carboxylation reaction appears to be carbon dioxide (Cooper *et al.*, 1968) and the sharp decrease in activity with increasing pH is possibly associated with the availability of this substrate. As the pH increases, the actual concentration of carbon dioxide at any fixed total concentration of bicarbonate will decrease. The activities of the cytosol and mitochondrial enzymes from monkey liver were optimal at pH 6.8 and 7.0 respectively, as compared with pH optima of 6.6 for pig liver mitochondrial PEPCK (Chang *et al.*, 1966), 7.4 for the cytosol and mitochondrial enzymes from guinea pig liver (Holten and Nordlie, 1965), 7.0 for the cytosol enzyme from adult rat liver and the mitochondrial enzyme of foetal rat liver (Ballard

and Hanson, 1967) and 6.2 for the chicken liver mitochondrial enzyme (Utter *et al.*, 1954; Utter and Kolenbrander, 1972).

For the decarboxylation reaction, the pH optimum for monkey liver cytosol PEPCK (7.0 - 8.0) was somewhat broader than for the carboxylation reaction and was comparable with the optimal pH range (7.3 - 8.1) reported for the enzyme from rat liver cytosol (Foster *et al.*, 1967). The activity of the monkey liver mitochondrial enzyme was optimal at pH 7.0 - 7.2 whereas optimal pH ranges of 6.6 - 8.0, 8.0 - 8.4 and 7.0 - 8.0 have been reported for the enzyme from the mitochondria of pig, guinea pig, and chicken livers, respectively (Utter *et al.*, 1954; Holten and Nordlie, 1965; Chang *et al.*, 1966; Utter and Kolenbrander, 1972). The cytosol enzyme from guinea pig liver, however, had a sharper pH optimum at pH 8.2 (Holten and Nordlie, 1965).

The variations in the pH optima of PEPCK from different species do not appear to result from the use of different buffer systems by different workers; in most cases (as in this study) imidazole-HCl or Tris-HCl buffers have been used for measurement of activity in the regions of optimal pH. Differences in pH optima depending on the metal ion used have, however, been reported for sheep kidney PEPCK (Barns and Keech, 1972).

When assayed in either direction at pH 7.0, the monkey liver enzymes were activated by manganese, iron II, and magnesium in order of decreasing effectiveness. The results obtained for iron II in the decarboxylation direction were not, however, considered very reliable, owing to the fact that the solutions absorbed at the wavelength at which phosphoenolpyruvate was determined. This may have resulted partly from formation of an iron II-oxaloacetate complex and partly from oxidation of iron II to iron III. Holten and Nordlie (1965) reported that when assayed in the carboxylation direction, both guinea pig liver enzymes

exhibited an absolute requirement for manganese, which could not be replaced by magnesium, calcium or zinc. In the decarboxylation direction, the guinea pig liver enzymes were activated by manganese and magnesium and to a lesser extent by iron II or cobalt II; at pH 7.3 both forms were more active with manganese than with magnesium, at all concentrations tested, but at pH 8.0, with metal-ion concentrations exceeding 4 mM, the mitochondrial enzyme was more active with magnesium (Holten and Nordlie, 1965).

The activities of the monkey liver enzymes reached a maximum when the manganese chloride:ITP (or GTP) ratio was 1:1, whereas for guinea pig liver cytosol and mitochondrial PEPCK and rat liver cytosol PEPCK the optimum ratio was 1:2 (Holten and Nordlie, 1965; Foster *et al.*, 1967). At manganese chloride concentrations exceeding those required for maximal activity, the monkey liver enzymes were inhibited, as was also shown to be the case for the guinea pig liver enzymes and the rat liver cytosol enzyme (Holten and Nordlie, 1965; Foster *et al.*, 1967).

The values obtained for the Michaelis constants for phosphoenolpyruvate of the monkey liver cytosol (0.14 mM) and mitochondrial (0.15 mM) enzymes were similar to those reported for guinea pig liver cytosol (0.14 mM) and mitochondrial (0.13 mM) PEPCK (Holten and Nordlie, 1965) and pig liver mitochondrial PEPCK (0.1 - 0.3 mM) (Chang *et al.*, 1966). Swiatek *et al.* (1970), however, reported a slightly higher value (0.6 mM) for the enzyme from pig liver mitochondria. The values obtained for the cytosol enzyme of adult rat liver and the mitochondrial enzyme of foetal rat liver were both 0.4 mM (Ballard and Hanson, 1967). The Michaelis constants for phosphoenolpyruvate are, in general, similar to or somewhat higher than the physiological concentration of this substrate, which is of the order 0.04 - 0.12 mM in rat liver (Hornbrook *et al.*, 1965).

The Michaelis constants of the monkey liver enzymes for bicarbonate (9.7 and 12.5 mM for the cytosol and mitochondrial forms, respectively) were comparable with those reported by Chang *et al.* (1966) (16 and 25 mM) and Swiatek *et al.* (1970) (15 mM) for the pig liver mitochondrial enzyme. These values are of a similar order of magnitude to the physiological concentration of bicarbonate (Long (1961) has reported a concentration of 20 mM for mammalian plasma).

It was originally considered that the bicarbonate ion was the functional species involved in the PEPCK-catalysed reaction (Chang *et al.*, 1966; Maruyama *et al.*, 1966), but as mentioned previously, it now appears from studies of bacterial PEPCK that carbon dioxide is in fact the actual substrate (Cooper *et al.*, 1968). The values reported here for the Michaelis constants of the monkey liver cytosol and mitochondrial enzymes for bicarbonate (determined at pH 7.0) correspond to values 1.9 and 2.5 mM, respectively, for carbon dioxide. For pig liver mitochondrial PEPCK, the values reported by Chang *et al.* (1966) (determined at pH 7.4) correspond to values 1.2 and 1.9 mM for carbon dioxide; the value obtained by Swiatek *et al.* (1970) (determined at pH 6.4) corresponds to a value 7.5 mM for carbon dioxide.

The values obtained for the Michaelis constants of the monkey liver cytosol and mitochondrial enzymes for IDP were 0.29 and 0.12 mM, respectively, as compared with values 0.033 - 0.098 mM reported for pig liver mitochondrial PEPCK (Chang *et al.*, 1966; Swiatek *et al.* 1970) and 0.07 - 0.62 mM reported for the guinea pig liver enzymes (Holten and Nordlie, 1965). The Michaelis constants of both the monkey liver enzymes for GDP (0.033 and 0.014 mM for the cytosol and mitochondrial forms, respectively) were considerably lower than those for IDP, whereas Chang *et al.* (1966) reported that the Michaelis constant of the pig liver

mitochondrial enzyme for GDP (0.063 mM) was within the range of values obtained for IDP. The intracellular concentration of GDP in rat liver is of the order 0.1 - 0.2 mM (Clifford *et al.*, 1972) and it therefore seems likely that *in vivo* the enzyme would be fully saturated with respect to this substrate.

In this study, the Michaelis constants of monkey liver cytosol and mitochondrial PEPCK for oxaloacetate were found to be 0.027 and 0.022 mM, respectively. The published values of this constant for PEPCK from tissues of other species vary considerably from one report to another. Values of 0.0015 - 0.005 (Walsh and Chen, 1971), 0.009 and 0.019 (Jomain-Baum *et al.*, 1976), 0.022 and 0.3 (under different conditions, see p.212) (Meyuhas *et al.*, 1971), 0.026 and 0.029 (Ballard, 1970), 0.14 (Foster *et al.*, 1967) and 0.15 mM (Marco and Sols, 1970) have been reported for rat liver cytosol PEPCK. The values 0.022, 0.1 and 0.9 mM were obtained for the enzyme from sheep liver cytosol (Ballard, 1970), mouse liver cytosol (Berndt and Ulbrich, 1970) and guinea pig liver cytosol (Holten and Nordlie, 1965), respectively. Ballard (1970) reported that the values for sheep and chicken liver mitochondrial PEPCK were 0.009 mM whereas Felicioli *et al.* (1970) obtained a value 4 mM for the chicken liver mitochondrial enzyme. The values 0.14 and 2.5 mM were obtained for the pig and guinea pig liver mitochondrial enzymes, respectively (Chang *et al.*, 1966; Holten and Nordlie, 1965).

It can be seen from the above, that the fairly low values obtained for the Michaelis constants of the monkey liver enzymes for oxaloacetate were of the same order of magnitude as values reported by Ballard (1970), Meyuhas *et al.* (1971) and Jomain-Baum *et al.* (1976) for the enzyme from other species. The higher values reported by others mentioned above are not easily reconciled with the physiological concentrations of oxaloacetate

(2-10 and 0.1-2 μM in the cytosol and mitochondrial compartments of rat liver, respectively) or with the observed rates of glucose synthesis (Williamson *et al.*, 1968, 1969; Scrutton and Utter, 1968).

There are several possible reasons for the wide range of values reported by different workers. The discrepancies could result partly from the use of a number of widely differing assay techniques (Pogson and Smith, 1975). The results obtained in this study were, however, comparable with results obtained by Ballard (1970), Meyuhas *et al.* (1971) and Jomain-Baum *et al.* (1976), who used different methods. It is also possible that PEPCK could exhibit co-operative behaviour with respect to oxaloacetate, giving non-linear double reciprocal plots. In this work no deviation from linearity was observed using oxaloacetate at concentrations between 3 and 100 μM. Similarly, Ballard (1970) found that the plots were linear when using oxaloacetate at concentrations ranging from 10 μM to 3 mM. Utter and Kolenbrander (1972) have, however, produced results which seem to indicate that under certain conditions, in which both manganese and magnesium ions are present, Michaelis-Menten kinetics are not observed. The major factor which could account for the differences in Michaelis constants, however, is the problem caused by the non-enzymic breakdown of oxaloacetate to pyruvate, which makes it difficult to determine the true oxaloacetate concentrations. In the present study, care was taken to prepare and assay oxaloacetate solutions immediately before use and initial velocity was determined as soon as the reaction had been initiated with oxaloacetate. Ballard (1970) stressed the importance of using freshly prepared oxaloacetate solutions and since he was using the Seubert-Huth assay, which is not a continuous method, he took the actual oxaloacetate concentration available for the PEPCK reaction as the average of the initial concentration and the final concentration (in the absence of enzyme) after the required incubation time. Meyuhas *et al.* (1971) found that when oxaloacetate

was added directly to the reaction mixture the value obtained for the Michaelis constant of rat liver cytosol PEPCK (0.3 mM) was considerably higher than when oxaloacetate was generated *in situ* from malate (0.022 mM). Systems in which oxaloacetate is generated *in situ* could, however, give rise to errors, since malate has been shown to be inhibitory (Ballard, 1970).

The Michaelis constant of the monkey liver cytosol enzyme for oxaloacetate was similar to that of the mitochondrial enzyme, whereas Ballard (1970) reported a value for the sheep liver cytosol enzyme which was 2-3-fold higher than that for the mitochondrial enzyme.

The respective values obtained for the Michaelis constants of the monkey liver cytosol and mitochondrial enzymes for ITP were 1.1 and 0.32 mM, as compared with published values of 0.6 - 1.8 mM for guinea pig liver cytosol and mitochondrial PEPCK (Holten and Nordlie, 1965), 0.58 mM for pig liver mitochondrial PEPCK (Chang *et al.*, 1966) and 2.9 mM for chicken liver mitochondrial PEPCK (Felicioli *et al.*, 1970). Walsh and Chen (1971), however, reported considerably lower values for the enzymes from rat liver cytosol (0.005 mM), guinea pig liver cytosol (0.004 mM) and guinea pig liver mitochondria (0.0015 mM).

The Michaelis constants of the monkey liver enzymes for GTP were somewhat lower than those for ITP, which is consistent with the observations of Chang *et al.* (1966) for pig liver mitochondrial PEPCK. Holten and Nordlie (1965) reported that the guinea pig liver enzymes had similar Michaelis constants for GTP and ITP. The values of the Michaelis constants of monkey liver cytosol and mitochondrial PEPCK for GTP were 0.16 and 0.13 mM, respectively, as compared with the reported values of 1.4 mM for the guinea pig liver cytosol and mitochondrial enzymes (Holten and Nordlie, 1965), 0.16 mM for pig liver mitochondrial PEPCK (Chang *et al.*, 1966) and 0.06 mM for the rat liver cytosol enzyme (Foster *et al.*, 1967). The

values obtained by Walsh and Chen (1971) were again considerably lower and were 0.005 mM for rat liver cytosol and guinea pig liver cytosol PEPCK and 0.0015 mM for guinea pig liver mitochondrial PEPCK. Jomain-Baum *et al.* (1976) also reported fairly low values (0.016 and 0.022 mM) for the rat liver cytosol enzyme.

The reason for the discrepancies between the values for the Michaelis constants for ITP and GTP reported by different workers is not clear. It is possible that erroneously high values could be obtained if the nucleoside triphosphates used contained the respective diphosphates which, in some species at least, inhibit the enzyme competitively with respect to nucleoside triphosphate (Chang *et al.*, 1966; Felicioli *et al.*, 1970). In the present study, ITP and GTP solutions were assayed before use and were not found to contain IDP or GDP, respectively.

The intracellular concentration of GTP is in the 0.1 - 0.6 mM range (Chance *et al.*, 1965; Clifford *et al.*, 1972); the Michaelis constants for this substrate reported here for monkey liver PEPCK and those reported by Holten and Nordlie (1965), Chang *et al.* (1966) and Foster *et al.* (1967) for the enzymes from other species fall within a similar range or are somewhat higher. This is consistent with a regulatory role for GTP, as has been suggested by Garber and Ballard (1970). The lower Michaelis constants obtained by Walsh and Chen (1971) and Jomain-Baum *et al.* (1976), however, suggest that the enzyme would be fully saturated with respect to GTP.

In an attempt to find possible regulators of monkey liver PEPCK activity, the effects of a number of compounds on the activities of the enzymes were tested. In the carboxylation direction, activity was determined either in the presence of manganese or iron II. In the

decarboxylation direction, activity was determined in the presence of manganese; iron II was not used owing to the fact that, as mentioned above, the assay was not very satisfactory

Substances which did not affect the enzyme activities significantly in either direction were AMP, a number of amino acids, α-ketoglutarate, succinate, malate and sodium fluoride. Others have studied the effects of these compounds on the enzymes from other species, but only in the decarboxylation direction. Utter and Kolenbrander (1972) showed that the chicken liver enzyme was unaffected by a variety of amino acids and Foster *et al.* (1967) found that α-ketoglutarate, succinate and malate had little effect on rat liver cytosol PEPCK. Ballard (1970), however, reported that the enzymes from chicken liver mitochondria, sheep liver cytosol and mitochondria and rat liver cytosol were inhibited by malate, but only at relatively high concentrations. Unlike the monkey liver enzymes, the guinea pig liver cytosol and mitochondrial forms are inhibited by AMP (Holten and Nordlie, 1965) and pig liver mitochondrial PEPCK is inhibited by fluoride (Chang and Lane, 1966).

Substances which were found to inhibit the monkey liver enzymes were IMP, IDP, GDP, ITP, GTP, citrate, isocitrate, hydrazine sulphate, tryptophan and quinolinic acid.

In the carboxylation direction, IMP inhibited both enzymes in the presence of either manganese or iron II. In the decarboxylation direction, the cytosol enzyme was somewhat inhibited whereas the mitochondrial enzyme was unaffected.

The inhibitory effects of ITP and GTP in the carboxylation direction and IDP and GDP in the decarboxylation direction were presumably due to binding of these substances with the metal-ion activator. When

iron II replaced manganese in the carboxylation reaction, the effects were considerably reduced, probably because iron II binds less strongly than manganese. In the decarboxylation direction, both enzymes were inhibited by GDP but only the cytosol form was significantly affected by IDP. Similarly, Chang and Lane (1966) showed that the pig liver mitochondrial enzyme was inhibited by GDP but not by IDP when assayed in the direction of phosphoenolpyruvate synthesis. Felicioli *et al.* (1970), however, found that the chicken liver mitochondrial enzyme was inhibited by IDP.

The monkey liver enzymes were both inhibited by citrate and isocitrate, when assayed in either direction. In the carboxylation direction, citrate inhibited the enzymes to a lesser extent in the presence of iron II than in the presence of manganese. The effects of citrate and isocitrate were probably due to metal-ion chelation, since the activity was found to increase if the manganese chloride was increased. Citrate and isocitrate have been reported to be without effect on rat liver cytosol PEPCK (Foster *et al.*, 1967).

Hydrazine sulphate did not inhibit the carboxylation reaction and inhibited only the cytosol enzyme in the direction of phosphoenolpyruvate synthesis. This compound has also been shown to inhibit rat liver cytosol PEPCK, in the decarboxylation direction (Ray *et al.*, 1970).

It was interesting that, although tryptophan had no effect on either of the monkey liver enzymes in the presence of manganese, the mitochondrial enzyme was inhibited in the presence of iron II in the carboxylation direction. However, the significance of this is not clear at present.

In the carboxylation direction, in the presence of manganese,

quinolinic acid inhibited both of the monkey liver enzymes to a similar extent; in the presence of iron II, the effect was much reduced. In the decarboxylation direction, the cytosol enzyme was inhibited to a greater extent than the mitochondrial form. Robinson and Oei (1975), however, reported that quinolinic acid had a similar effect on the cytosol and mitochondrial enzymes from guinea pig liver. Snoke *et al.* (1971) found that the iron II-activated rat liver cytosol enzyme was inhibited by quinolinic acid, but that the manganese-activated enzyme was not. It has been suggested that a tryptophan-quinolinate control mechanism is operative in rat liver. It was found that administration of tryptophan to rats resulted in increased hepatic PEPCK activity even though the rate of gluconeogenesis decreased (Foster *et al.*, 1966b; Ray *et al.*, 1966; Foster *et al.*, 1967). This was explained by the formation of quinolinate during the metabolism of tryptophan. It was suggested that an iron II-quinolinate complex, a strong inhibitor of PEPCK, was formed after administration of tryptophan, but that in preparing the tissue for assay of PEPCK *in vitro*, the complex was sufficiently diluted so that the enzyme was left in a metal-activated state (Snoke *et al.*, 1971).

Of the gluconeogenic enzymes, PEPCK is perhaps one of the most interesting. It is unique in that its distribution in the liver cell varies greatly among species; this has important implications concerning the mechanism and control of hepatic gluconeogenesis in different animals. The majority of earlier studies of PEPCK have involved non-primate species. In this work, the subcellular distribution of PEPCK in monkey liver was examined and the cytosol and mitochondrial enzymes were characterised. Comparison of the properties of the monkey liver enzymes showed that there were some differences between the two forms. The properties of the

monkey liver enzymes also differed to some extent from those reported for the enzymes from other species. At present, however, insufficient information is available concerning primate liver PEPCK, and the physiological significance of such differences is not yet clear. Further studies aimed at obtaining a better understanding of the somewhat complex regulatory and catalytic properties of PEPCK, in species such as the monkey, would therefore be of interest. It is also possible that studies of this and other enzymes in primate tissues would provide a more suitable model for the human situation than studies involving more distantly-related species.

CHAPTER IV

KINETIC STUDIES OF BABOON LIVER ALKALINE PHOSPHATASE

SUMMARY

1. Alkaline phosphatase was purified from the livers of baboons which had undergone auxiliary transplants and had raised levels of the enzyme.

2. Some of the kinetic properties of the purified enzyme, which had a final specific activity of 166 U/mg, were studied.

3. The Michaelis constants of the enzyme for p-nitrophenyl phosphate in the presence of 0.76 M Ammediol buffer at pH 9.0 and 10.17 were 0.044 and 0.44 mM, respectively. Similar values (0.04 and 0.51 mM at pH 9.0 and 10.17, respectively) were obtained for a less highly purified preparation (specific activity 17.8 U/mg). The Michaelis constant for phenolphthalein monophosphate at pH 9.9 increased from 0.97 to 2.71 mM as the molarity of the buffer (2-amino-2-methylpropan-1-ol) decreased from 0.57 to 0.3 M. The Michaelis constants of baboon serum alkaline phosphatase for p-nitrophenyl phosphate at pH 10.17 and phenolphthalein monophosphate at pH 9.9 were 0.49 and 1.07 mM, respectively.

4. L(+)-Homoarginine, L(+)-arginine, L(+)-lysine, L(+)-leucine, imidazole, L(-)-phenylalanine and L(+)-alanine inhibited the enzyme uncompetitively, affecting both the maximum velocity and apparent Michaelis constant. L(+)-Homoarginine was the most potent inhibitor and the other compounds are listed in order of decreasing inhibitory capacity.

5. L(+)-Cysteine and L(-)-histidine were both found to be non-competitive inhibitors, affecting maximum velocity only and not the Michaelis constant. L-Cysteine was a very strong inhibitor (K_i = 0.077 mM).

6. The enzyme was activated by magnesium ions, with a slight fall in activity at magnesium concentrations greater than 0.4 mM, and inhibited by zinc ions after an initial slight activation at a zinc concentration of 0.08 mM.

7. The inhibitory effect of EDTA was complex, remaining constant up to 10 mM EDTA concentration, after which the activity was further reduced. The effect was also time-dependent. Partial reactivation was brought about by addition of magnesium ions.

8. After incubation at 60° for 20 minutes only 11% of the activity remained, and at 58° the enzyme was 50% inactivated after 32 minutes.

9. On isoelectric focusing a single band from pH 4.3 - 4.6 was found.

10. Except for its greater thermostability, the baboon liver alkaline phosphatase resembled human liver alkaline phosphatase in all parameters tested.

METHODS

Chemicals

p-Nitrophenyl phosphate was obtained from E. Merck, Darmstadt, Germany and phenolphthalein monophosphate as "Phosphastrate alkaline" concentrated buffered substrate from General Diagnostics, Warner-Lambert Co., Morris Plains, New Jersey, U.S.A. 2-Amino-2-methylpropan-1-ol and 2-amino-2-methylpropan-1,3-diol (Ammediol) were obtained from British Drug Houses, Poole, England. The amino acids L(-)-phenylalanine, L(+)-alanine, L(-)-histidine and L(+)-lysine monohydrochloride were obtained from E. Merck; L(+)-cysteine hydrochloride, L(+)-arginine hydrochloride and imidazole (glyoxaline) from British Drug Houses; L(+)-leucine from Sigma Chemical Co., St. Louis, Missouri, U.S.A. and L(+)-homoarginine hydrochloride from Calbiochem, San Diego, California, U.S.A. TEAE-cellulose was from Serva, Heidelberg, Germany and Sephadex G-200 from Pharmacia, Uppsala, Sweden. Ampholines were obtained from LKB, Uppsala, Sweden. β-Naphthyl phosphate and Fast Blue BB salt were from Sigma. Other standard chemicals were obtained from British Drug Houses, Poole, England, E. Merck, Darmstadt, Germany and Hopkin and Williams, Chadwell Heath, England, and were of analytical grade.

Tissue

Livers obtained from chacma baboons *(Papio ursinus)* which had undergone auxiliary transplant operations were supplied by Dr. J. Jersky. These livers contained approximately 3 - 10 times more alkaline phosphatase activity than livers from normal animals. Serum was also obtained from a baboon which had undergone an auxiliary liver transplant.

Alkaline phosphatase assays

p-Nitrophenyl phosphate as substrate

A continuous spectrophotometric method similar to that described by Bowers and McComb (1966) was used. The formation of p-nitrophenol was followed at 30° and 404 nm using a Beckman DB spectrophotometer.

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow[\text{phosphatase}]{\text{alkaline}} \text{p-nitrophenol} + P_i$$

The reaction mixture contained 0.76 M 2-amino-2-methylpropan-1,3-diol (Ammediol) buffer, pH 10.17, 4 mM p-nitrophenyl phosphate and enzyme in a total volume of 3 ml. Ammediol buffer has been shown to give optimal activities compared with other buffer systems (Bowers and McComb, 1966). The reaction was initiated by addition of enzyme. Enzyme was omitted from the blank.

A unit of enzyme activity is defined as the amount of enzyme catalysing the production of 1 µmole of p-nitrophenol per minute at 30°, using a value for the molar extinction coefficient of p-nitrophenol at 404 nm of 18.7 (Bowers and McComb, 1966).

Phenolphthalein monophosphate as substrate

The formation of phenolphthalein was followed continuously at 37° and 550 nm, using a Beckman DB spectrophotometer.

$$\text{phenolphthalein monophosphate} + H_2O \xrightarrow[\text{phosphatase}]{\text{alkaline}} \text{phenolphthalein} + P_i$$

Since phenolphthalein monophosphate is unstable and was only obtainable commercially as "Phosphastrate alkaline" substrate already dissolved in 2-amino-2-methylpropan-1-ol buffer, pH 10.15, it was diluted

to give the required final concentrations with this same buffer, which has been shown to give optimal activities compared with other buffers of the same pH value (Babson *et al.*, 1966).

The assay mixture, which was similar to that described by Babson *et al.* (1966), contained 0.3 or 0.57 M 2-amino-2-methylpropan-1-ol buffer, pH 10.15, substrate at the required concentration and enzyme in a total volume of 1 ml. The actual pH at 37° was 9.9. The reaction was initiated by addition of enzyme. Enzyme was omitted from the blank.

Protein determination

Proteins were estimated using the method of Warburg and Christian (1942), in which optical densities of solutions were determined at 260 and 280 nm and a correction applied to account for nucleic acid concentration.

PURIFICATION PROCEDURE

Alkaline phosphatase was purified from baboon livers using the method of Engström (1964). In all cases centrifugation was carried out at 4°, using either an MSE 18 High Speed refrigerated centrifuge or a Sorvall SS-3 centrifuge which was kept in the cold room.

Extraction of tissue

A mixture of four donor and recipient livers of total mass 1.4 kg was used. The livers were cut into small pieces and homogenised, at room temperature, in batches of 125 g. Each 125 g of liver was homogenised in a mixture of 125 ml n-butanol and 375 ml 10 mM sodium acetate - 10 mM magnesium acetate, using a Waring blender. The homogenates were combined and after 1 hour the suspension was centrifuged at 1,000 g for 10 minutes.

The supernatant consisted of an upper butanol layer which was siphoned off and discarded and a lower aqueous layer which contained alkaline phosphatase activity. The aqueous layer was recentrifuged and the remaining butanol was siphoned off and discarded. The tissue residue was re-extracted by homogenising in 3 litres of 10 mM sodium acetate - 10 mM magnesium acetate and then centrifuging as before. The aqueous extracts were combined.

Acetone and ethanol fractionations

Acetone and ethanol fractionations were performed at room temperature. The required amounts of solvent were added to the enzyme preparations and after 1 hour, protein precipitates were collected by centrifugation at 15,000 g for 20 minutes.

Acetone precipitation (50%)

An equal volume (2,390 ml) of acetone was added to the combined aqueous tissue extracts. The protein precipitate was collected and enzyme activity was recovered by extracting with magnesium acetate. The precipitate was homogenised with 240 ml 0.5 M magnesium acetate and the suspension was centrifuged. The procedure was repeated using a further 240 ml 0.5 M magnesium acetate. The extracts were combined.

First ethanol fractionation (30 - 75%)

Ethanol was added to the combined extracts to give a final concentration of 30%. The protein precipitate was discarded. The ethanol concentration of the solution was increased to 75%. The protein precipitate was collected and dissolved in 250 ml 10 mM magnesium acetate.

Second ethanol fractionation (30 - 55%)

The enzyme was once more fractionated with ethanol. Ethanol was added to the solution to give a final concentration of 30%. The protein precipitate was discarded. The ethanol concentration of the solution was increased to 55%. The protein precipitate was collected and dissolved in 92 ml of 0.5 M magnesium acetate.

Acetone fractionation (30 - 55%)

After the second ethanol fractionation, acetone was added to the solution to give a final concentration of 30%. The protein precipitate was discarded. The acetone concentration of the solution was increased to 55%. The protein precipitate was collected and dissolved in 12 ml 10 mM Tris-acetic acid, pH 8.8. The solution was dialysed against 10 mM Tris-acetic acid, pH 8.8 containing 5 mM magnesium acetate for 24 hours.

Column chromatography

TEAE-Cellulose was prepared as described by Himmelhoch (1971). It was washed on a Buchner funnel with 0.5 M sodium chloride - 0.5 N sodium hydroxide, rinsed with distilled water and then washed with 0.5 N hydrochloric acid. This procedure was repeated three times. After a final wash with 0.5 M sodium chloride - 0.5 N sodium hydroxide, the TEAE-cellulose was rinsed thoroughly with distilled water and was then equilibrated with starting buffer. Sephadex G-200 was equilibrated with the appropriate buffer for 3 - 4 days (with at least three changes of buffer during this time) and then washed extensively on a Buchner funnel with the same buffer.

Chromatography was carried out in the cold room at 4°. Packed columns were equilibrated with at least two volumes of starting buffer. Column effluents were passed through an LKB 6250H Uvicord absorptiometer

(attached to a chopper bar recorder), which gave an optical density reading at 254 nm, and then collected using an LKB 3401B fraction collector.

First TEAE-cellulose column

After the 30 - 55% acetone fractionation step, the dialysed enzyme (15 ml) was applied to a TEAE-cellulose column (5 cm^2 x 25 cm) which had been equilibrated with 10 mM Tris-acetic acid, pH 8.8 containing 5 mM magnesium acetate. The column was washed with the same buffer until the unadsorbed protein had been removed. Elution was carried out using 800 ml of a linear gradient of 10 mM Tris-acetic acid, pH 8.8 - 5 mM magnesium acetate and 50 mM Tris-acetic acid, pH 8.8 - 100 mM magnesium acetate. The flow rate was 0.8 ml/minute and the volume of each fraction collected was 6.7 ml. The active fractions were combined. Solid ammonium sulphate was added to bring the solution to 70% saturation at 0° (100% saturation = 70 g ammonium sulphate/100 ml solution). The solution was stirred gently for 30 minutes. The precipitated protein was then collected by centrifugation at 31,000 g, 4°, for 20 minutes and dissolved in 18 ml 10 mM Tris-acetic acid, pH 8.0.

Second TEAE-cellulose column

The concentrated eluate from the first TEAE-cellulose column was dialysed against 10 mM Tris-acetic acid, pH 8.8 for 24 hours. The dialysed enzyme was then applied to a second TEAE-cellulose column (5 cm^2 x 25 cm) which had been equilibrated with 10 mM Tris-acetic acid, pH 8.8. The column was washed with the same buffer until the unadsorbed protein had been removed. Elution was carried out using 800 ml of a linear gradient of 10 mM Tris-acetic acid, pH 8.8 and 50 mM Tris-acetic acid, pH 8.8 - 250 mM sodium acetate. The flow rate was 0.8 ml/minute

and the volume of each fraction collected was 7 ml. The active fractions were combined and the solution was brought to 70% saturation at 0° with solid ammonium sulphate. After 30 minutes, the protein precipitate was collected by centrifugation as above and dissolved in 8 ml 25 mM Tris-acetic acid, pH 8.8 containing 1 M sodium acetate.

Sephadex G-200 chromatography

The concentrated TEAE-cellulose eluate was applied to a Sephadex G-200 column (0.65 cm^2 x 60 cm), which had been equilibrated with 25 mM Tris-acetic acid, pH 8.8 containing 1 M sodium acetate. The enzyme was eluted with the same buffer at a flow rate of 0.1 ml/minute. The volume of each fraction collected was 3 ml. The active fractions were combined and stored in the deep freeze at -20°.

KINETIC STUDIES

Before kinetic studies were carried out the purified enzyme preparation was dialysed against the appropriate assay buffer for at least 1 hour and then diluted with 0.1% albumin solution, in order to eliminate a lag in the reaction rate, which was apparent when the enzyme was diluted in water or buffer. Concentrations of substrates, inhibitors and metal ions in individual experiments were as indicated in the Results. All assays were carried out in duplicate.

Reciprocal velocities were plotted against reciprocal substrate concentrations (Lineweaver and Burk, 1934). Apparent Michaelis constants and their standard errors were determined as described previously (p.184), using the computer programme of Cleland (1963). Weighted means of the Michaelis constants and their standard errors were obtained as described by Morrison and Uhr (1966). Data conforming to uncompetitive and non-

competitive inhibition were fitted to equations (1) and (2), respectively, using the appropriate computer programmes of Cleland (1963).

$$v = \frac{VA}{K + A\left(1 + \frac{I}{K_{ii}}\right)} \qquad (1)$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \qquad (2)$$

where v is the observed velocity, V is the maximum velocity, K is the apparent Michaelis constant, A is the substrate concentration, I is the inhibitor concentration and K_{ii} and K_{is} are the inhibitor constants affecting the slope and intercept, respectively.

ISOELECTRIC FOCUSING

Isoelectric focusing was carried out as described in Chapter II (p.121), using Ampholines in the pH range 3 - 10.

After removal from the running tubes, the gels were stained for alkaline phosphatase activity using the β-naphthyl phosphate, Fast Blue BB stain of Smith *et al.* (1968) with the addition of zinc sulphate, in order to overcome partially the chelating effects of the Ampholines. The stain buffer, 60 mM boric acid - 10 mM magnesium chloride, pH 9.7, contained 8 mM β-naphthyl phosphate, 5 mM zinc sulphate and 1 mg Fast Blue BB/ml. β-Naphthyl phosphate was omitted from the blank.

The pH gradient was determined, using unstained gels, as described previously (p.122).

RESULTS

The purification of alkaline phosphatase from baboon livers involved acetone and ethanol fractionations and TEAE-cellulose and Sephadex G-200 chromatography. The results obtained during these procedures are given in Table I.

There were no detectable activity losses on storing the purified enzyme either at 4° or -20° over a period of three months.

Kinetic studies

Some of the kinetic properties of baboon liver alkaline phosphatase were examined. Unless otherwise stated, the final purified enzyme preparation, of specific activity 166 U/mg, was used.

Apparent Michaelis constants

The apparent Michaelis constants of the enzyme for p-nitrophenyl phosphate and phenolphthalein monophosphate are shown in Table II. Very similar Michaelis constants for p-nitrophenyl phosphate, in the presence of 0.76 M Ammediol buffer, pH 10.17, were found for the enzyme at two different stages of the purification procedure (the final purified preparation of specific activity 166 U/mg and a preparation of specific activity 17.8 U/mg purified to the end of the second ethanol fractionation step). A similar value was also obtained for serum from a baboon which had undergone auxiliary liver transplantation. Using the same buffer at pH 9.0, the Michaelis constants of both alkaline phosphatase preparations for p-nitrophenyl phosphate were found to be considerably lower. The Michaelis constant of the enzyme of highest specific activity for phenolphthalein monophosphate in 2-amino-2-methylpropan-1-ol buffer, pH 9.9, increased as the molarity of the buffer decreased from 0.57 to 0.3 M. The Michaelis constant of serum

TABLE I

PURIFICATION OF BABOON LIVER ALKALINE PHOSPHATASE

PURIFICATION STAGE	VOLUME (ml)	TOTAL ACTIVITY (U)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %	PURIFICATION FACTOR PER STEP	OVERALL PURIFICATION FACTOR
Crude extract	2390	43020	25573	1.68	100	1	1
Acetone precipitation (50%)	500	21875	7250	3.02	51	1.8	1.8
First ethanol fractionation (30 - 75%)	260	20150	2080	9.69	47	3.2	5.8
Second ethanol fractionation (30 - 55%)	100	18000	1010	17.8	42	1.9	11
Acetone fractionation (30 - 55%)	15	12900	528	24.4	30	1.4	15
First TEAE-cellulose eluate (concentrated)	20	3800	140	27.1	8.8	1.1	16
Second TEAE-cellulose eluate (concentrated)	10	2600	30	86.7	6	3.2	52
Sephadex G-200 eluate	24	2400	14.5	166	5.6	1.9	100

TABLE II

APPARENT MICHAELIS CONSTANTS OF BABOON LIVER ALKALINE PHOSPHATASE

SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE PREPARATION	SUBSTRATE	BUFFER		pH	K_m (mM)
166	p-Nitrophenyl phosphate	Ammediol	0.76 M	10.17	0.44 ± 0.01*
17.8				10.17	0.51 ± 0.06
Serum				10.17	0.49 ± 0.03
166				9.0	0.044 ± 0.003
17.8				9.0	0.040 ± 0.004
166	Phenolphthalein monophosphate	2-Amino-2-methyl-propan-1-ol	0.57 M	9.9	0.97 ± 0.12
166			0.3 M	9.9	2.71 ± 0.45
Serum			0.3 M	9.9	1.07 ± 0.04

p-Nitrophenyl phosphate concentrations were varied from 0.166 - 1 mM at pH 10.17 and from 0.0166 - 0.1 mM at pH 9.0. Phenolphthalein monophosphate concentrations were varied from 0.416 to 2.5 mM. Michaelis constants and standard errors were calculated using the appropriate computer programme of Cleland (1963).

*Weighted mean of ten determinations

alkaline phosphatase for this substrate was somewhat lower than that of the purified enzyme.

Inhibition by amino acids

The mechanism of inhibition by the amino acids listed in Table III was investigated by varying the substrate concentration at several fixed inhibitor concentrations. Velocities were determined at 30° with p-nitrophenyl phosphate concentrations varying from 0.166 to 1 mM in the presence of 0.76 M Ammediol buffer, pH 10.17, containing the inhibitors at the final concentrations shown in Table III.

When reciprocal velocities were plotted against reciprocal substrate concentrations at various concentrations of inhibitor, a series of parallel straight lines was obtained for L(+)-alanine, L(-)-phenylalanine, imidazole, L(+)-leucine, L(+)-lysine, L(+)-arginine and L(+)-homoarginine, showing that these substances were all uncompetitive inhibitors, changing both apparent Michaelis constant and maximum velocity. The plots obtained using L(+)-alanine and imidazole are shown in Figs. 1 and 2, respectively. The other amino acids at the appropriate concentrations gave similar plots. The values of the inhibitor constants (K_{ii}) calculated for each of these substances are given in Table III. It can be seen that in many cases the inhibitor constant was dependent on the concentration of inhibitor and generally decreased with increasing inhibitor concentration. The strongest inhibitor in the uncompetitive group was L(+)-homoarginine and then in decreasing order, L(+)-arginine, L(+)-lysine, L(+)-leucine, imidazole, L(-)-phenylalanine and L(+)-alanine.

The baboon liver alkaline phosphatase was inhibited differently by L(-)-histidine and L(+)-cysteine. The lines obtained on plotting reciprocal velocities against reciprocal substrate concentrations at

TABLE III

INHIBITOR CONSTANTS OF BABOON LIVER ALKALINE PHOSPHATASE

FOR SEVERAL L-AMINO ACIDS AND IMIDAZOLE

INHIBITOR	INHIBITOR CONCENTRATION (mM)	K_{ii} (mM)	TYPE OF INHIBITION
L(+)-Alanine	200	134.2 ± 6.9	Uncompetitive
	300	93.5 ± 4.4	
L(-)-Phenylalanine	20	46.4 ± 2.8	
	40	16.7 ± 0.7	
	60	16.1 ± 0.5	
Imidazole	2	13.4 ± 2.5	
	8	19.5 ± 2.2	
	16	15.4 ± 0.9	
L(+)-Leucine	10	12.0 ± 0.6	
L(+)-Lysine	5	9.14 ± 0.78	
	10	8.47 ± 0.63	
	20	9.24 ± 0.48	
L(+)-Arginine	10	5.36 ± 0.26	
L(+)-Homoarginine	2	2.15 ± 0.28	
	4	1.78 ± 0.14	
	8	1.20 ± 0.05	
L(-)-Histidine	10	13.3 ± 2.6	Non-competitive
	20	8.07 ± 1.65	
L(+)-Cysteine	0.04	0.077 ± 0.008	

The assay mixture contained 0.76 M Ammediol buffer, pH 10.17, inhibitor, and p-nitrophenyl phosphate at concentrations between 0.166 and 1 mM. Inhibitor constants and standard errors were calculated using the appropriate computer programme of Cleland (1963).

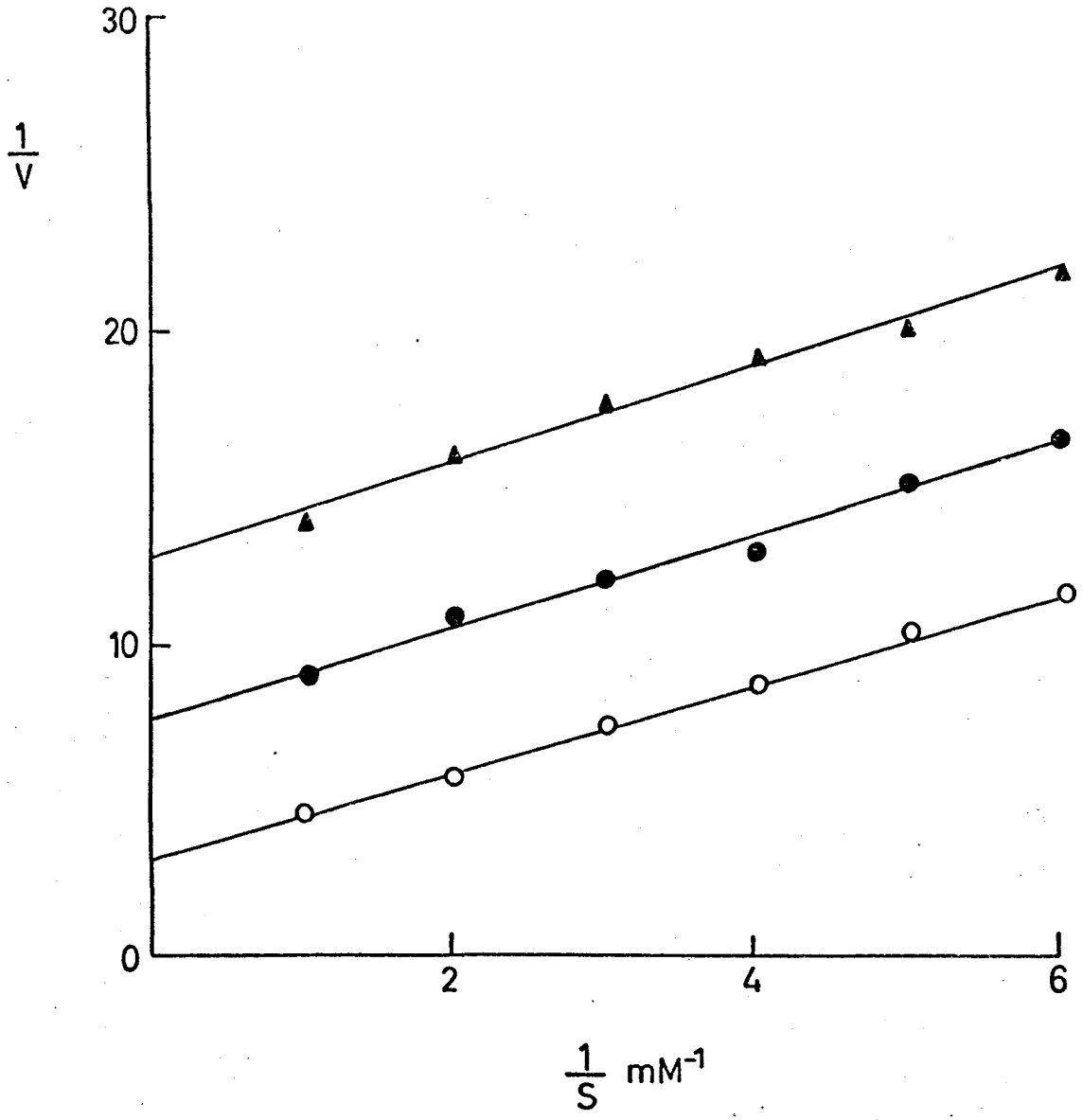


FIGURE 1. Double reciprocal plots of velocity against substrate concentration at different concentrations of L(+)-alanine for baboon liver alkaline phosphatase. p-Nitrophenyl phosphate concentration was varied from 0.166 to 1 mM in 0.76 M Ammediol buffer, pH 10.17. L(+)-Alanine concentrations were 0 mM (o), 100 mM (●) and 200 mM (▲).

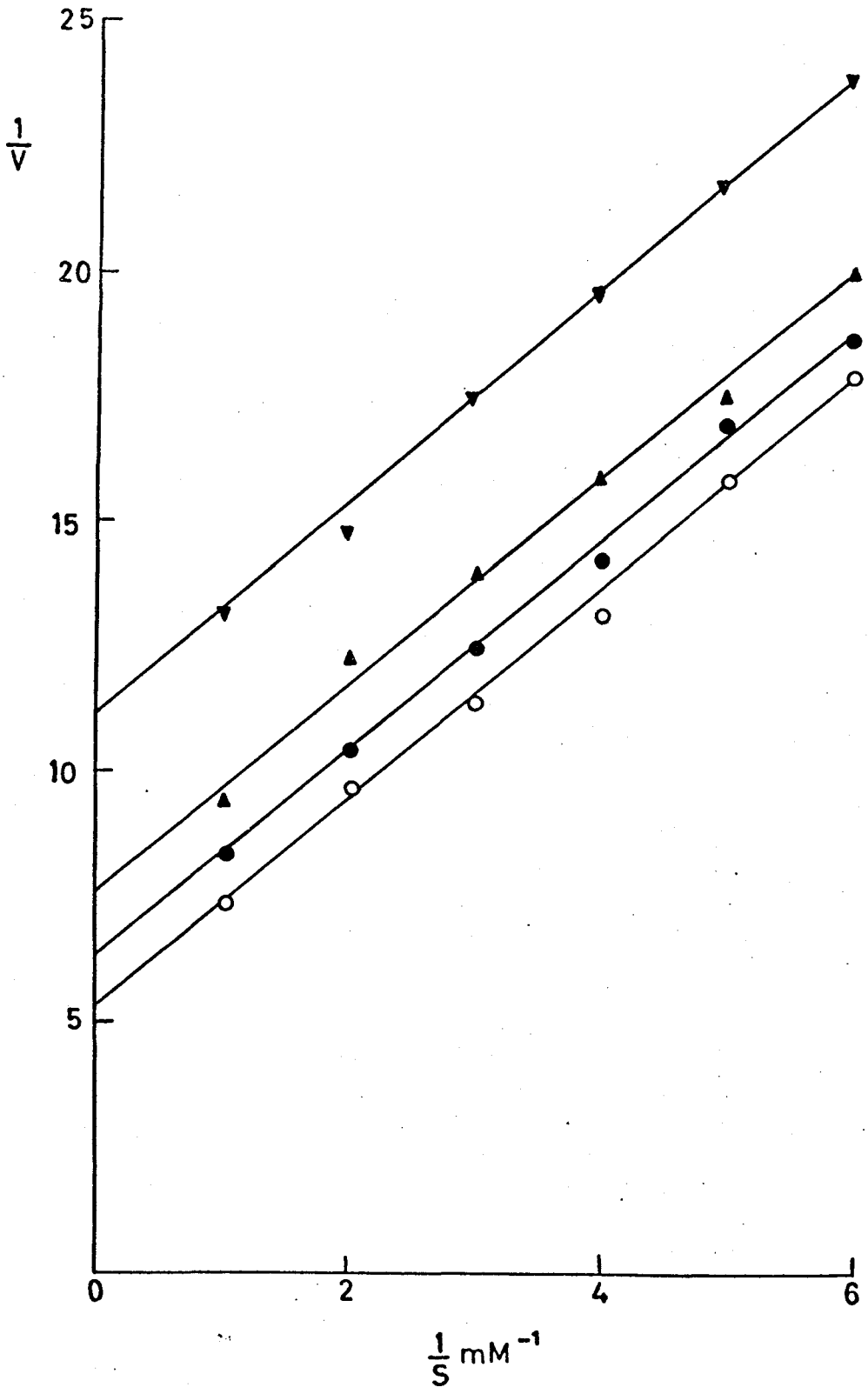


FIGURE 2. Double reciprocal plots of velocity against substrate concentration at different concentrations of imidazole for baboon liver alkaline phosphatase. p-Nitrophenyl phosphate concentration was varied from 0.166 to 1 mM in 0.76 M Ammediol buffer, pH 10.17. Imidazole concentrations were 0 mM (o), 2 mM (●), 8 mM (▲) and 16 mM (▼).

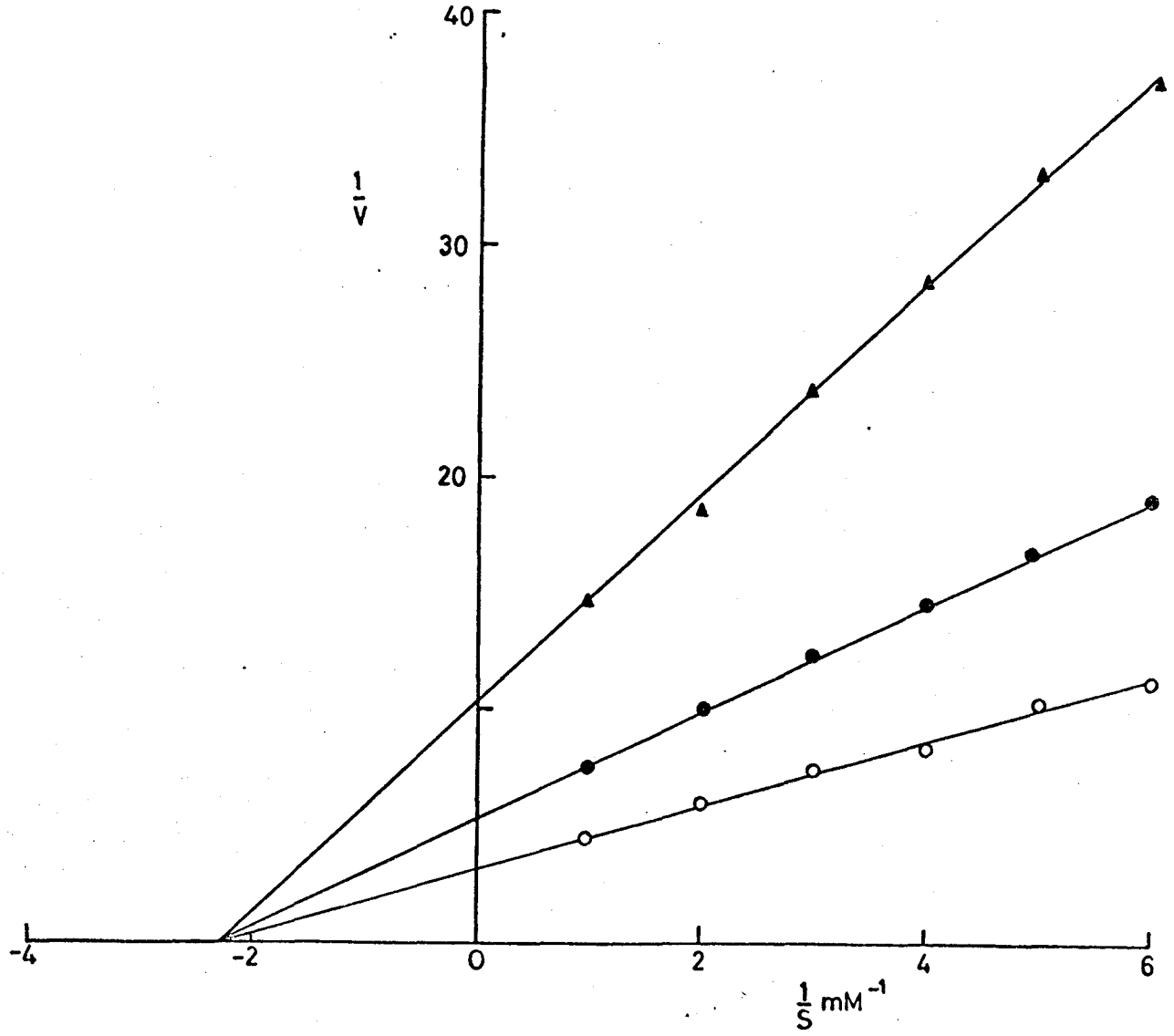


FIGURE 3. Double reciprocal plots of velocity against substrate concentration at different concentrations of L(-)-histidine for baboon liver alkaline phosphatase. p-Nitrophenyl phosphate concentration was varied from 0.166 to 1 mM in 0.76 M Ammediol buffer, pH 10.17. Histidine concentrations were 0 mM (o), 10 mM (●) and 20 mM (▲).

various concentrations of inhibitor converged and met at a point on the abscissa corresponding to $-\frac{1}{K_m}$, showing that these two substances were non-competitive inhibitors, affecting maximum velocity only and not the Michaelis constant. The plot for L(-)-histidine is shown in Fig. 3. From this plot the inhibitor constant for 10 mM L(-)-histidine was found to be 13.3 mM. L(+)-Cysteine was a very strong non-competitive inhibitor with an inhibitor constant of 0.077 mM.

Effect of magnesium and zinc ions

The effects of varying concentrations of magnesium and zinc ions on the activity of baboon liver alkaline phosphatase, determined at 30° in the presence of 4 mM p-nitrophenyl phosphate and 0.76 M Ammediol buffer pH 10.17, are shown in Fig. 4.

When magnesium chloride was included in the assay system, the activity increased between 0 and 0.2 mM magnesium chloride, remained constant between 0.2 and 0.4 mM magnesium chloride, and then dropped gradually as the concentration was increased to 4 mM, though it was still greater than the value in the absence of magnesium.

When zinc sulphate replaced magnesium chloride in the assay system, the activity rose as the concentration of zinc sulphate increased from 0 to 0.08 mM, but the activation was less than with 0.08 mM magnesium chloride. Beyond 0.08 mM zinc sulphate concentration, the activity fell and at 0.2 mM had reached the level obtained in the absence of added zinc. At 1 mM zinc sulphate concentration, the activity was only 36% of the activity with no zinc sulphate.

Varying the zinc sulphate concentration in the presence of 0.1 mM magnesium chloride resulted in activity loss at all zinc sulphate

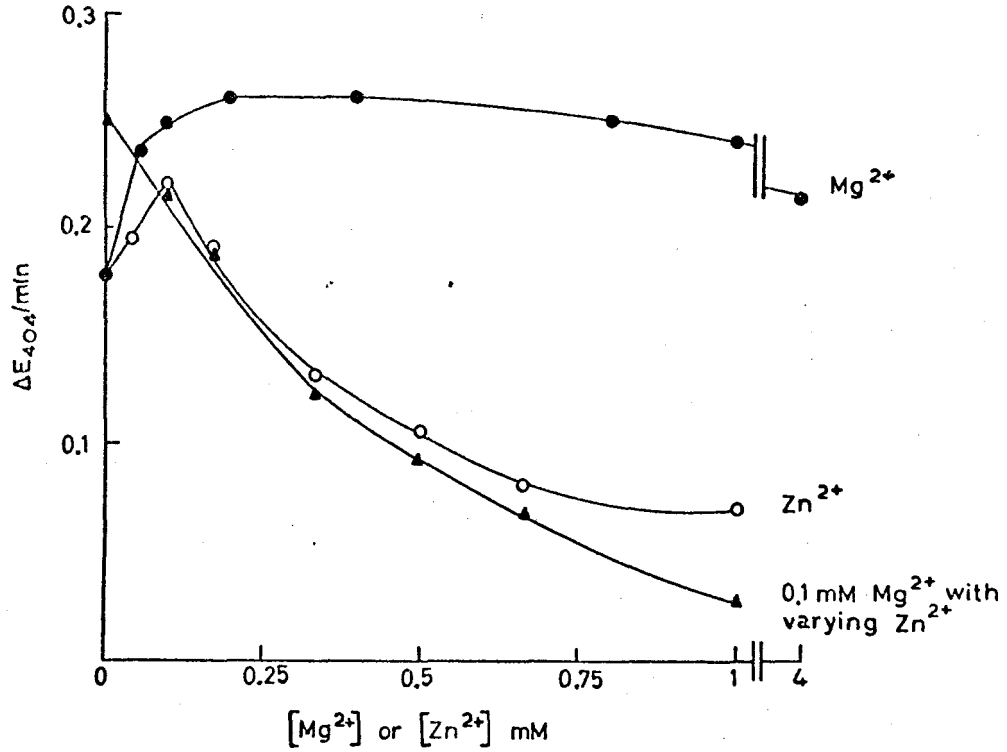


FIGURE 4. Effect of varying concentrations of zinc and magnesium ions on the activity of baboon liver alkaline phosphatase. The substrate was 4 mM p-nitrophenyl phosphate in 0.76 M Ammediol buffer, pH 10.17.

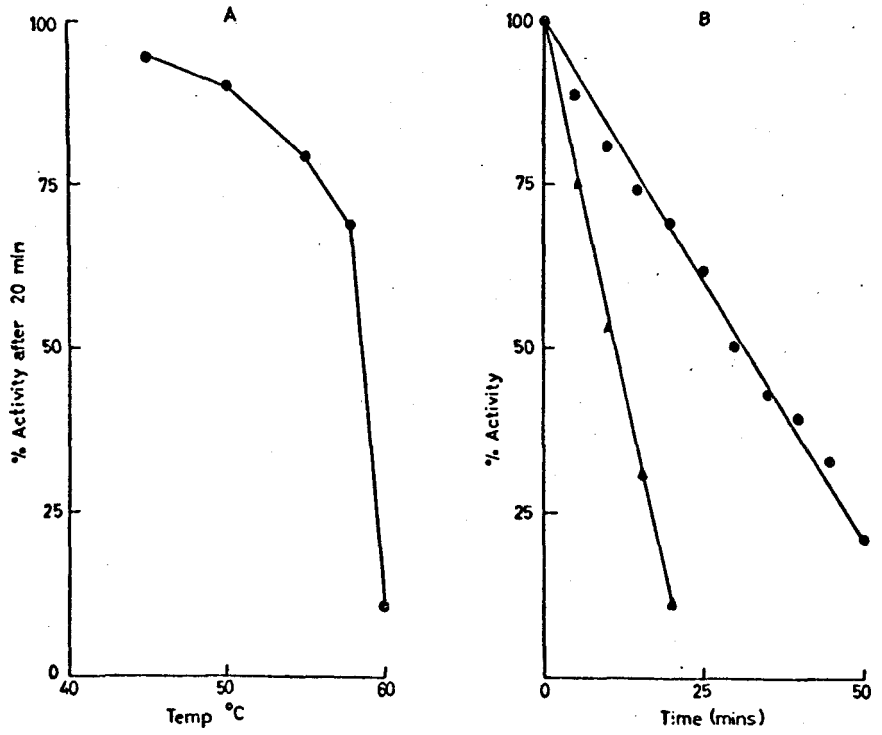


FIGURE 5. Heat stability of baboon liver alkaline phosphatase. The substrate was 4 mM p-nitrophenyl phosphate in 0.76 M Ammediol buffer, pH 10.17. A. Effect of incubating the enzyme for 20 minutes at various temperatures. B. Change of enzyme activity with time at 58° (●) and 60° (▲).

concentrations tested.

Effect of EDTA

Enzyme activity was assayed using 1 mM p-nitrophenyl phosphate as substrate in 0.76 M Ammediol buffer, pH 10.17, containing various concentrations of EDTA, both in the absence of magnesium ions and with 0.1 mM magnesium chloride present in the assay system. As shown in Table IV, EDTA caused approximately 20% loss of activity at concentrations between 0.05 and 10 mM when assayed without magnesium, followed by steady drop as the EDTA concentration was increased to 100 mM. At concentrations of EDTA of 10 mM and higher, the activity loss was considerably greater when 0.1 mM magnesium chloride was included in the assay system.

The enzyme was preincubated at 30° with different concentrations of EDTA in Ammediol buffer, pH 10.17, and the residual activity was measured at varous time intervals. It can be seen from Table IV that, following incubation with 0.2 mM EDTA for 30 minutes, the activity was the same either with or without magnesium chloride in the assay system. At higher concentrations of EDTA, no activity remained after 10 minutes when the enzyme was assayed without magnesium, whereas with magnesium present, 91% of the original activity could be detected even at 2.5 mM EDTA, presumably owing to reactivation in the cuvette. With 3 mM EDTA and magnesium in the cuvette only 17% of the activity was measurable after 10 minutes.

Thermostability studies

Enzyme activity was determined at 30° in the presence of 0.76 M Ammediol buffer, pH 10.17 and 4 mM p-nitrophenyl phosphate. The enzyme was

TABLE IV

EFFECT OF EDTA ON BABOON LIVER ALKALINE PHOSPHATASE

EDTA (mM)	TIME OF PREINCUBATION (mins)	% REMAINING ACTIVITY	
		ASSAYED WITHOUT Mg^{2+}	ASSAYED IN PRESENCE of 0.1 mM Mg^{2+}
0	0	100	100
0.05	0	84	85
1	0	83	82
10	0	79	30
50	0	41	17
100	0	24	15
0.2	10	87	92
	20	79	78
	30	66	67
0.5	5	24	91
	10	0	84
	15	0	74
2.5	5	0	96
	10	0	91
3.0	5	0	24
	10	0	17

The assay system contained 1 mM p-nitrophenyl phosphate in 0.76 M Ammediol buffer, pH 10.17. In the first experiment EDTA at the indicated concentration was added directly to the assay cuvette. In all other experiments an aliquot of the preincubation mixture containing EDTA at the indicated concentration, was diluted 30-fold in the assay cuvette at the times shown.

then incubated at several different temperatures for 20 minutes and re-assayed. The results obtained are shown in Fig. 5A. It can be seen that after 20 minutes incubation at 45°, 95% of the activity remained, but that at 60°, only 10% of the activity was retained in the same period of time.

The activity was also measured at 5 minute intervals during incubation at 58° and 60°. It can be seen from Fig. 5B, that there was a steady loss of activity at both 58° and 60°, with greater activity loss at 60° at each time interval measured.

Isoelectric focusing

On isoelectric focusing, using Ampholines in the pH range 3 - 10, the purified enzyme gave a single band in the pH region 4.3 - 4.6. Dialysis prior to staining was not found to enhance the intensity of the bands appreciably, but caused them to diffuse and consequently to stain over a wider area.

DISCUSSION

Alkaline phosphatase has been studied in a wide variety of species and there is considerable evidence for the existence of both organ- and species-specific variants of the enzyme (for reviews see Fishman and Ghosh, 1967; Fernley, 1971). Detailed studies of alkaline phosphatases from human tissues have been carried out and the different isozymes are well characterised (Moss and King, 1962; Fishman and Ghosh, 1967; Fishman *et al.*, 1968; Eaton and Moss, 1968; Moss, 1971). Marked differences have, however, been shown between the properties of placental alkaline phosphatase from human subjects and from other primates (Manning *et al.*, 1969b, 1970). In this work, a study of alkaline phosphatase purified from the liver of baboons which had undergone auxiliary transplants was undertaken. As a result of transplantation, the levels of the enzyme in donor livers were approximately three times greater than in normal livers and in recipient livers up to ten times greater than in normal livers (Jersky, 1974).

The Michaelis constants of baboon liver alkaline phosphatase for p-nitrophenyl phosphate were 0.044 mM at pH 9.0 and 0.44 mM at pH 10.17. These values are of the same order of magnitude as the values 0.06 mM and 0.8 mM at pH 9.0 and 10.5, respectively, reported for this substrate for the enzyme from human liver by Eaton and Moss (1968). Variations of both Michaelis constant and maximum velocity relative to pH have also been shown for alkaline phosphatases from human intestine, placenta and bone, using either p-nitrophenyl phosphate or phenyl phosphate as substrate (Fishman and Ghosh, 1967; Fishman *et al.*, 1968; Eaton and Moss, 1968).

The Michaelis constants of the baboon liver enzyme for phenolphthalein monophosphate were higher than those for p-nitrophenyl phosphate and the values obtained depended on the molarity of the buffer. The enzymes

from human liver and placenta have also been shown to have different Michaelis constants for several different substrates (Fishman and Ghosh, 1967; Harkness, 1968b; Moss, 1971).

Several amino acids (L(+)-homoarginine, L(+)-arginine, L(+)-lysine, L(+)-leucine, L(-)-phenylalanine and L(+)-alanine) and imidazole were found to be uncompetitive inhibitors of the baboon liver enzyme. In many cases it was found that the inhibitor constant was dependent on the concentration of inhibitor and generally decreased with increasing inhibitor concentration. The most potent uncompetitive inhibitor studied was L(+)-homoarginine, with an inhibitor constant of 1.2 - 2.15 mM. This compound is a potent uncompetitive inhibitor of human liver alkaline phosphatase, with an inhibitor constant of 4.5 mM (wrongly calculated as 0.22 mM, Lin and Fishman, 1972; these workers plotted reciprocal maximum velocities against L-homoarginine concentrations and took the intercept on the abscissa to be $1/K_i$, instead of $-K_i$). L-Homoarginine also inhibits human bone alkaline phosphatase, but has virtually no effect on the intestinal or placental isozymes (Fishman and Sie, 1970). L(+)-Arginine was also quite a potent inhibitor (K_i 2.15 - 5.36 mM) of the baboon liver enzyme followed by L(+)-lysine, L(+)-leucine and imidazole in order of decreasing strength. Human liver alkaline phosphatase has also been shown to be inhibited by L(+)-arginine, L(+)-lysine and imidazole; the human placental enzyme is, however, affected to a much lesser extent by these compounds (Fishman and Sie, 1970; Brunel and Cathala, 1972). L(-)-Phenylalanine and L-(+)-alanine were weak uncompetitive inhibitors of the baboon liver enzyme. L-Phenylalanine is also a weak uncompetitive inhibitor of the human liver enzyme (Fishman *et al.*, 1963; Ghosh and Fishman, 1966, 1968; Fishman and Ghosh, 1967). This amino acid has virtually no effect on baboon or monkey placental alkaline

phosphatases (Manning *et al.*, 1969b) but is a strong inhibitor of the human placental and intestinal isozymes (Ghosh and Fishman, 1966; Fishman and Ghosh, 1967). The inhibition of human alkaline phosphatases by L-phenylalanine has been shown to be stereospecific and non-allosteric (Ghosh and Fishman, 1966).

Fernley and Walker (1970) have provided evidence to show that L-phenylalanine inhibits human placental alkaline phosphatase by preventing the breakdown of a phosphoryl-enzyme following release of the non-phosphate product. It would be reasonable to assume that other uncompetitive inhibitors act in a similar way, the extent of inhibition depending on structural differences between the placental, liver and other isozymes. Since the baboon liver enzyme was inhibited by the compounds tested to a similar extent as the human liver enzyme, the two are probably structurally similar at the active site.

Baboon liver alkaline phosphatase was found to be inhibited non-competitively by L(+)-cysteine and L(-)-histidine. L(+)-Cysteine was a very strong inhibitor and the inhibitor constant obtained (0.077mM) was similar to that reported by Agus *et al.* (1966) for the human kidney enzyme (0.085 mM). These workers have shown that alkaline phosphatase preparations from a number of different tissues and species are inhibited to similar extents by L-cysteine and that the mechanism of inhibition of the human kidney enzyme is non-competitive and non-stereospecific. They suggest that the inhibition is a result of chelation *in situ* with zinc atom at the active site. It would appear that the zinc atom in the baboon liver enzyme is as accessible as in other alkaline phosphatases. The effect of L-histidine, which has also been reported to be a non-stereospecific inhibitor of the enzyme from tissues of several other species (Brunel and Cathala, 1972), is probably similar to that of L-cysteine.

The activation of baboon liver alkaline phosphatase by magnesium ions and the slight fall of activity at higher concentrations (probably an ionic strength effect) have been observed for alkaline phosphatases from tissues of other species (Fernley, 1971). It is thought that magnesium activates the enzyme by increasing the number of active sites rather than by enhancing a particular rate (Fernley and Walker, 1970). The inhibitory effect of zinc, following slight activation, has been reported previously for other alkaline phosphatases (Fernley, 1971); the presence of magnesium in addition to zinc did not reactivate the baboon liver enzyme.

Inhibition of the baboon liver enzyme by EDTA was similar to that described by Conyers *et al.* (1967) for the human bone and intestinal enzymes; the instantaneous inhibition is independent of variations in EDTA concentration up to about 10 mM, and beyond that the activity drops. The greater drop in activity in the presence of magnesium ions could be attributed to a conformational change at the active site. The time-dependent inactivation by EDTA has also been reported by Conyers *et al.* (1967) and Harkness (1968b) for human tissue phosphatases. Since EDTA is a chelating agent, it probably removes the functionally essential zinc atom, by binding *in situ* at the active site; magnesium in the assay mixture produces partial reactivation.

The baboon liver alkaline phosphatase appeared to be more stable than the human liver enzyme. The baboon liver enzyme was only 20% inactivated after 20 minutes at 55°, whereas the human liver enzyme was found to be inactivated by 50% after 18 minutes at the same temperature (Moss and King, 1962). After 16 minutes at 55°, the baboon and monkey placental isozymes were reported to be 61 - 67% inactivated, whereas the human placental isozyme had lost only 1 - 2% of the initial activity (Manning *et al.*, 1969b, 1970).

The single band obtained on isoelectric focusing of baboon liver alkaline phosphatase was at pH 4.3 - 4.6. This value is lower than the value of pH 5.0 - 5.5 obtained for human liver alkaline phosphatase by Smith *et al.* (1971) using the same technique. Latner *et al.* (1970) and Trépanier *et al.* (1976), using sucrose density gradient isoelectric focusing, obtained values of 3.9 and 4.0, respectively, for the isoelectric point of the human liver enzyme.

The studies carried out by Manning *et al.* (1969b) showed that baboon placental alkaline phosphatase differed from human placental alkaline phosphatase in possessing greater thermolability and being less inhibited by L-phenylalanine. From the present study of alkaline phosphatase from baboon liver, it can be seen that this enzyme is less thermolabile than the human liver enzyme, suggesting some structural differences. The pattern of inhibition for the human liver enzyme is closely simulated by the baboon liver enzyme, implying a similarity in the nature of the catalytic site and suggesting that in this case the effects are organ-specific as opposed to species-specific.

SUMMARY

I. A comparative study of the activities of the key gluconeogenic enzymes, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) and the key enzyme of glycogen synthesis, glycogen synthetase (GS) in rat and human livers, hepatomas and hepatoma cell cultures was carried out. In both rat and human hepatomas, changes in the activities of these enzymes, possibly indicative of reduced rates of gluconeogenesis and glycogen synthesis, as compared with normal or host livers, were observed. In human hepatomas, the levels of PC, PEPCK and G6Pase were similar to those of human foetal livers, whereas the levels of GS were somewhat lower than in foetal livers. The levels of PC, PEPCK and G6Pase in the rat and human hepatoma cells were, in general, similar to or lower than those of the respective tumours; GS activities were often higher in the hepatoma cells than in the hepatomas.

II. The isozyme patterns of phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PD), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) in rat and human muscle, livers, hepatomas and hepatoma cell cultures were compared. The HK, PK and LDH isozyme patterns in human foetal tissues, other human tumour tissues (oesophageal cancer, meningioma, mesothelioma) and cell cultures (oesophageal cancer, HeLa) were also examined. The PGM and G6PD isozyme patterns in rat and human hepatomas were similar to those of liver. In both rat and human hepatomas, liver-specific HK and PK isozymes were frequently reduced or even absent; this suggests a loss of the differentiated functions with which these isozymes are associated in normal

liver. The LDH isozyme patterns in rat hepatoma were similar to those of liver; in human hepatoma occasional differences were observed. The HK, PK and LDH isozyme patterns in human hepatoma and other tumours showed some resemblance to those of foetal tissues. The isozyme patterns in the various cell cultures were not truly representative of those of the tissues of origin, but were, in general, characteristic of the patterns acquired by rapidly-dividing cells growing in culture.

III. Phosphoenolpyruvate carboxykinase was found to be almost equally distributed between the mitochondrial and cytosol fractions of monkey liver. The monkey liver enzymes were purified and some of their catalytic properties were examined. Studies of nucleoside phosphate specificity, pH optima, effects of divalent metal ions, Michaelis constants for the various substrates and effects of possible modulators, in the carboxylation and decarboxylation directions, were carried out. Differences between the mitochondrial and cytosol enzymes with respect to relative activities with GDP and CTP, pH optima in the decarboxylation direction, relative activities with manganese and magnesium in both directions, Michaelis constants for IDP, GDP and ITP and inhibitory effects of IDP, GDP, tryptophan, hydrazine sulphate and quinolinic acid were observed. The properties of the monkey liver enzymes differed to some extent from those reported for the enzymes from non-primate species.

IV. Alkaline phosphatase was purified from the livers of baboons which had undergone auxiliary transplant operations and had raised levels of the enzyme. Some of the kinetic properties of the purified enzyme were studied. The Michaelis constants for p-nitrophenyl phosphate and phenolphthalein monophosphate were determined and the

effects of amino acids, EDTA, divalent metal ions and heat were examined. The baboon liver enzyme was somewhat less thermolabile than human liver alkaline·phosphatase, but in all other respects studied, its properties resembled those reported for the human liver enzyme.

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