CONTROL OF SERINE DEHYDRATASE ACTIVITY IN RAT LIVER

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I declare that the dissertation which is herewith submitted for the degree of Master of Science in the University of the Witwatersrand is entirely my own work and that it has not been previously submitted for a degree in any other University

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June, 1980

ABSTRACT

Gluconeogenesis from amino acids in the liver is enhanced when the utilisation of glucose is limited under various hormonal and dietary conditions, such as diabetes, starvation or administration of a carbohydrate free diet. Pyruvate is of great importance as a carbon source for gluconeogenesis, since the sequence of gluconeogenic reaction is initiated by carboxylation of pyruvate to oxaloacetate.

From this point of view, an important physiological role is suggested for serine dehydratase, which catalyses the degradation of serine to pyruvate and ammonia. The relationship of serine dehydratase levels to gluconeogenic activities, however, is poorly understood. A study of the hormonal and dietary control of serine dehydratase activity was carried out *in vivo* and *in vitro* in rat liver.

Serine dehydratase was assayed by the colorimetric method of Suda and Nakagawa (1971) and the enzymatic method of Wimhurst and Manchester (1973). Both these methods have been found to be suitable since they are in agreement with each other and also give results which compare favourably with other published values. Activities of serine dehydratase from fresh liver and in slices of liver cultured for various periods have been compared. Also a study of the activity of another soluble enzyme, lactic dehydrogenase, was undertaken and the *in vivo* and *in vitro* levels were compared.

The present study has shown that serine dehydratase activity increased markedly on overnight starvation, in agreement with the findings of Suda (1967). Hydrocortisone injection had no significant inducting effect on eithor serine dehydratase or lactic dehydrogenase in normal and adrenalectomised rats, however diet does seem to play an important regulatory role. The activity of serine dehydratase is increased by injection of triiodothyronine to previously adrenalectomised and thyroidectomised rats. Injections of hydrocortisone and triiodothyronine to adrenalectomised and thyroidectomised rats doubled activity. This was in accordance with published literature.

The addition of hydrocortisone or β-methasone to the culture medium containing slices of normal and adrenalectomised rat liver which were cultured for 24 to 48 hours at 37°C did not significantly induce the activity of serine dehydratase. On comparison with the control levels there was a significant decrease in enzyme activity. On incubation of the slices serine dehydratase and lactic dehydrogenase activity fell markedly during the first 4 hours. Thereafter it remained fairly constant up to 24 hours. ·tic dehydrogenase activity in the medium steadily increased to a maximum at 12 hours, indicating that the enzyme had leaked out of the cell, but there was no accumulation of serine dehydratase. In view of the obvious big loss of enzyme from the tissue and the very low enzyme activity observed in the slices it is questionable

whether any physiological meaning can be attached to these changes.

Thirteen-day old rats were also assayed for serine dehydratase activity in order to see if the rate of decrease in enzyme activity on incubation was the same as in the adult and also whether steroids and other hormones have any significant effect. Serine dehydratase was induced by β-methasone on incubation from 24 to 48 hours at 37°C, and also by dbcAMP and on comparison to the adult rat the induction is twice as much in the younger animal.

Glugagon decreases serine dehydratase on its own but in the presence of dbcAMP there is an induction over a 48 hour incubation. Actinomycin D inhibits serine dehydratase activity by 33% on its own and 40% in the presence of dbcAMP.

That the organ culture system was able to maintain tissue in a viable state for at least 24 hours has been shown by the criteria of light and electron microscopy and the estimation of potassium ions.

VI

TO MY GRANDPARENTS, MOTHER AND WIFE

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ADR	–	Adrenalectomised
BSA	–	Bovine serume albumin
dbcAMP	–	Dibutyryl adenosine 3',5'-cyclic monophosphate
HC	–	Hydrocortisone
LDH	–	Lactic Dehydrogenase
NAD	–	Nicotinamide adenine dinucleotide
NADH	–	Nicotinamide adenine dinucleotide (reduced form)
N.S.	–	Not significant
SDH	–	Serine Dehydratase
THY	–	Thyroidectomised

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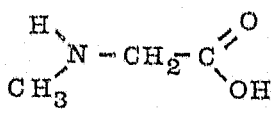
I INTRODUCTION

1. Serine Metabolism:-

Synthesis of the amino acid in the body of the vertebrate is limited to those that are not essential in the diet. Serine being a non-essential amino acid is synthesized in the body of various vertebrates and also by micro-organisms and plants. Serine is a component of most proteins and is a direct precursor of glycine, free methyl groups, phospholipids and cysteine. Therefore regulation of the biosynthesis of this essential intermediary metabolite is likely to be multifactorial.

Serine can be formed by the additions of a β carbon to glycine through transfer of a hydroxymethyl group by way of a formylated tetrahydrofolic acid. Sarcosine (N-methyl-glycine) may be considered to serve as the source of the hydroxymethyl group from which the β carbon originates (Harper 1973). The conversion of serine to glycine involves the loss of a single carbon, the β carbon, an active one carbon moiety which can be used for methylation or for purine synthesis (Scheme 1). Decarboxylation of serine yields ethanolamine. Since this compound on methylation forms choline, serine may be considered to participate in the formation of lipotropic agents. A cephalin fraction containing serine has been isolated from the brain (Harper, 1973). The compound phosphatidylserine, a phospholipid, is also found in the blood in traces.



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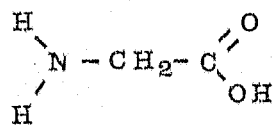
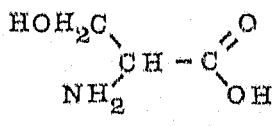
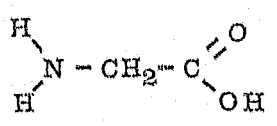


GLYCINE



SERINE



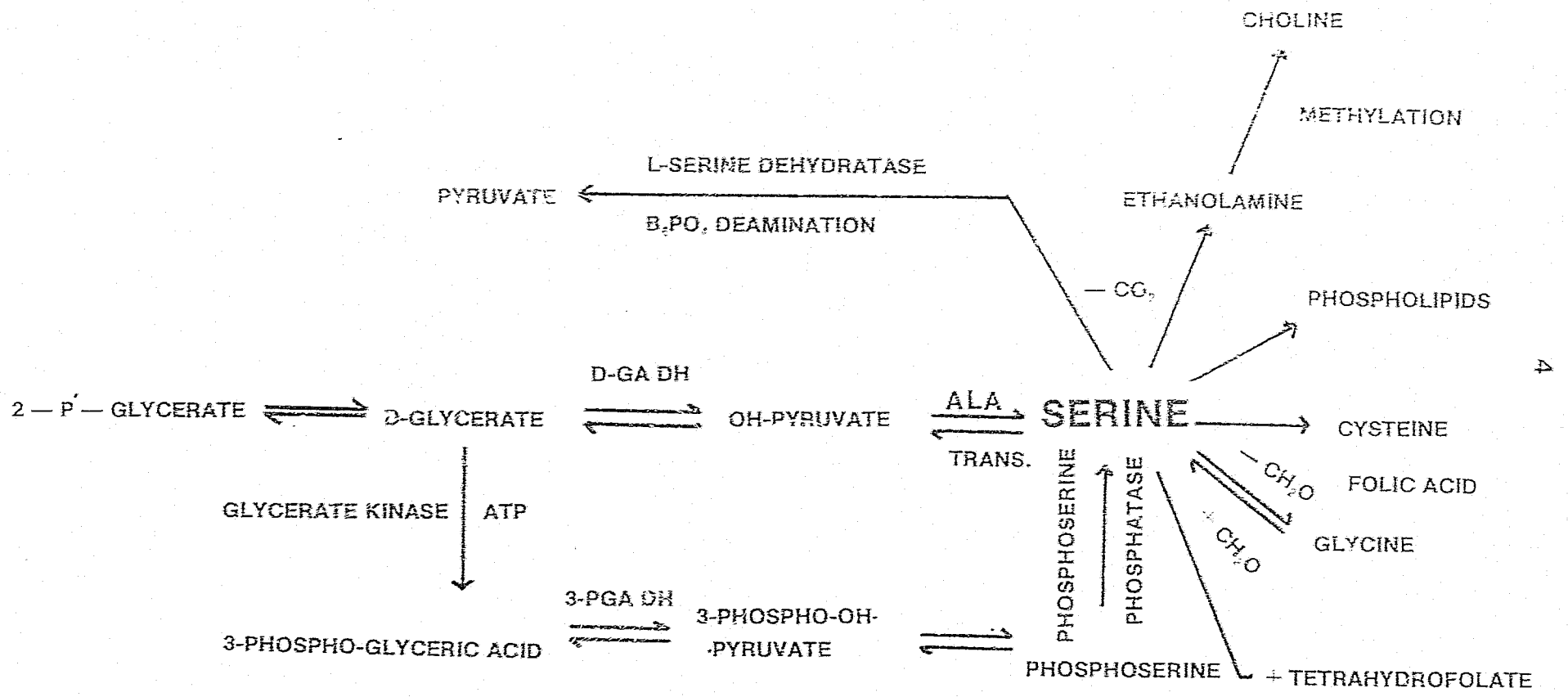
GLYCINE

SCHEME 1

Serine is also involved in the synthesis of sphingol and therefore in the formation of sphingomyelins of brain. It also participates in purine and pyrimidine synthesis. The β-carbon is a source of the methyl groups of thymine (and of choline) and of the carbons in positions 2 and 8 in the purine nucleus (Harper, 1973).

Two pathways are known for the synthesis of serine from carbohydrate precursors (Greenberg, 1969). Initial evidence was presented by Sallach for the formation of serine from D-glyceric acid the non-phosphorylated pathway (Sallach, 1956). Later Ichihara and Greenberg (1957) synthesised serine via 3-phospho-D-glyceric acid or the phosphorylated pathway. The two pathways are found to coexist in liver and kidneys of a variety of vertebrate species (Walsh and Sallach, 1966) and in Neurospora (Sojka and Garner, 1967). This is shown by the presence of the two chief enzymes of each pathway, D-glycerate dehydrogenase and hydroxypyruvate L-alanine aminotransferase for the non-phosphorylated pathway, and D-glycerate 3-phospho-dehydrogenase and 3-phosphohydroxypyruvate L-glutamate transaminase for the phosphorylated pathway (Walsh and Sallach, 1966). According to earlier reports of Sallach and Willis (1962) and Ichihara and Greenberg (1955, 1957) the non phosphorylated pathway is the major route of serine synthesis in the livers of the dog, rat and frog; the phosphorylated pathway appears to predominate in the liver of other vertebrate species and in the kidneys and brains of all vertebrate species.

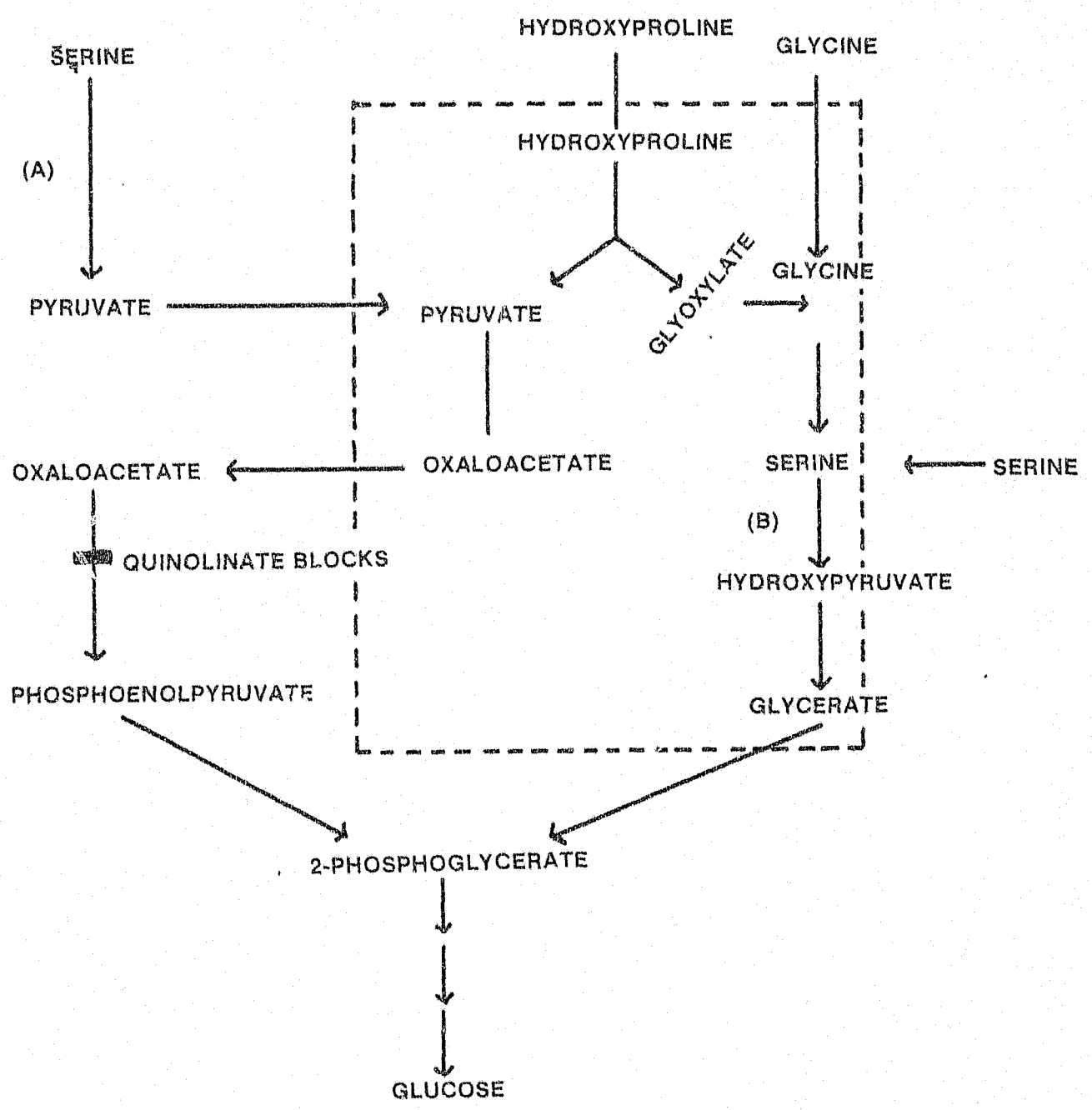
METABOLISM OF SERINE



SCHEME 2

It is accepted that the activity of gluconeogenesis from amino acids in the liver is enhanced where the utilisation of glucose is limited under various hormonal and dietary conditions such as diabetes, starvation or the administration of a carbohydrate-free diet, and pyruvate is considered to be of great importance as a carbon source for gluconeogenesis (Ishikawa et al., 1965). Since the sequence of gluconeogenic reactions is initiated by carboxylation of pyruvate to oxaloacetate (Henning et al., 1963; Wagle, 1963; Shrago et al., 1963), an important physiological role is suggested for serine dehydratase (E.C. 4.2.1.13), which catalyses the degradation of serine to pyruvate and ammonia (Ishikawa et al.,1965). According to Snell (1974) serine is an effective glucogenic precursor in adult rat liver. The route by which serine is converted into glucose is nevertheless the matter of some controversy. The alternative pathways (A) via pyruvate and initiated by L-serine dehydratase and (B) via hydroxypyruvate and initiated by L-serine-pyruvate amino transferase (E.C. 2.6.1.51) are outlined in scheme 3.

Much evidence suggests that the activity of L-serine dehydratase is increased in vivo under conditions associated with enhanced gluconeogenesis (Snell and Walker, 1973). Also the enhanced gluconeogenic capacity of the neonatal rat observed by Snell agrees well with previous findings in the whole animal in vivo (Walker and Snell, 1973) and with liver slices in vitro (Ballard and Oliver, 1963; Yeung and Oliver, 1963; Vernon et al., 1968). In considering the physiological



SCHEME 3

Alternative pathways for gluconeogenesis from L-serine (A) via L-serine dehydratase and (B) via L-serine-pyruvate aminotransferase

The pathways of potential amino acid precursors of L-serine are also shown. The dashed line encompasses reactions taking place predominantly intramitochondrially. For the sake of clarity possible mechanisms for the transfer of oxaloacetate out of the mitochondrion are not indicated.

significance of the alternative routes of glucose synthesis from serine (Scheme 3), it may be relevant that in the rat the pathway via pyruvate is predominantly cytosolic in sub-cellular location whereas the amino transferase pathway is located intramitochondrially (Rowsell et al., 1972). This raises the possibility that the amino-transferase pathway may be involved in gluconeogenesis from a mitochondrial serine pool and possible intramitochondrial sources of serine have been considered (Snell, 1974).

The dehydratase conversion of L-serine into pyruvate (and ammonia) need not serve exclusively as a starting point for glucose formation (Rowsell et al., 1973). The acute dependence of L-serine dehydratase activity on body size in adult mammals (Rowsell et al., 1965, Rowsell et al., 1972) and the striking increase that occurs in rats well supplied with carbohydrate but exposed to cold (Rowsell et al., 1965; Nakagawa and Nagai, 1971) suggest a role for this enzyme other than in gluconeogenesis. Even under conditions of carbohydrate shortage, a function for example in the provision of oxaloacetate for the TCA (Krebs) cycle could be of importance. The fact remains that a pathway from pyruvate to glucose is thoroughly established, so that to some degree it appears L-serine dehydratase must contribute to glucose formation, and an elevation of its activity under circumstances associated with gluconeogenesis is at least consistent with such a role (Rowsell et al., 1973).

The existence of multiple molecular forms of enzymes and other proteins

is now a well known and rather common phenomenon (Harris, 1968; Shaw, 1969). However, relatively few studies have been carried out on the effects of environmental regulation on the level of various isozymes. The finding of Goodfriend et al. (1966) that the presence of oxygen appears to 'induce' the heart form of lactate dehydrogenase is one example of such environmental control of a specific molecular form of an enzyme. The presence of isozymes in liver as compared to other tissues has demonstrated that in many instances the organ appears to have its own unique set of isozymes when compared to other tissues of the mammal. Many of the glycolytic enzymes have a specific liver form as opposed to other forms throughout the organism (Sols, 1965; Bigley et al., 1968). Some enzymes are found only in the liver, for example, serine dehydratase and tryptophan pyrrolase.

2. Serine Dehydratase

L-serine dehydratase has been considered a group specific enzyme (E.C. 4.2.1.13), catalyzing both the threonine dehydratase and cystathione synthetase reactions since Greenberg et al. (Selim and Greenberg, 1959; 1960; Nagabhushanam and Greenberg, 1965) found that a purified preparation of serine dehydratase exhibited both these activities. But Nakagawa et al. (1969) found that serine dehydratase was entirely different from cystathione synthetase in rat liver, and has later shown quite clearly (Nakagawa et al., 1967; Inoue and Pitot, 1970; Nakagawa and Kimura, 1969) that rat liver serine dehydratase is a single protein not possessing

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any cystathionine synthetase activity as had been previously described (Nagabhushanam and Greenberg, 1965). Two isozyme forms of the rat liver enzyme have been separated by acrylamide gel electrophoresis as well as on DEAE-cellulose (Inoue and Pitot, 1970). The levels of the two isozyme forms are different under different hormonal and nutritional circumstances in the animal. Inoue et al. (1971) also crystallised serine dehydratase from rat liver and determined a molecular weight of 64,000. On seperation by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis they found two homogeneous proteins. The more electropositive I form was regulated by corticosteroids in adrenalectomised rats, whereas the more electronegative II form was regulated by glucagon (Inoue and Pitot, 1970; Inoue et al., 1971). A high protein diet or tryptophan administration resulted in roughly equal levels of each form of the enzyme (Inoue and Pitot, 1970). Simon et al. (1973) also purified serine dehydratase and also found two isozymes. Inoue et al. (1976) in their experiments on the purification of serine dehydratase found the two isozymes of the enzyme to be two different proteins

with different regulations. Two major regulating factors of serine dehydratase synthesis are thus cortisone and glucagon. The fact that each form is probably independantly regulated by a different hormone has many interesting implications in a study of multiple forms of other enzymes. In particular where more than one hormone or regulatory mechanism is involved, it may well be predicted that more than one form of the enzyme will also be involved (Inoue and Pitot, 1970).

3. Regulation of Serine Dehydratase

Studies by several workers have shown that the rat liver enzyme L-serine dehydratase can be induced by forced feeding of enzymatically hydrolysed casein (Peraino and Pitot, 1964; Jost et al., 1969) to rats previously maintained on a protein free diet. The level of the enzyme is high in the alloxan induced diabetic rat (Soling et al.,1969, Ishikawa et al., 1965). Enzyme activity is low in pancreatectomised rats and is not increased by fasting, force feeding of amino acids or hydrocortisone administration (Ishikawa and Nakajima, 1970; Nakajima et al., 1970). Serine dehydratase can be induced in the adrenalectomised rats by hydrocortisone (Jost et al., 1967; Soling et al., 1969), glucagon (Soling et al., 1969) or amino acid intubation (Pitot and Peraino, 1964; Jost et al., 1967.) Hydrocortisone may potentiate the stimulation by glucagon or cyclic AMP in the pancreatectomised rat (Ishikawa and Nakajima, 1970; Nakajima and Ishikawa, 1971). Pancreactectomy depletes the body not only of pancreatic hormones but also of exocrine enzymes which degrade endogenous protein in the intestine and supply amino acids to the liver during starvation (Nakajima et al., 1970). Nakajima et al., (1970) set up experiments to test whether the lowered activity of serine dehydratase in pancreatectomised rat liver is accompanied by reduction in the activities of other enzymes unique to amino acid degradation and gluconeogenesis. The changes in activity of the enzyme after pancreatectomy differed, but two distinct types of enzyme were recognised. The activities of

the one type decreased more or less below the normal level after pancreatectomy and the activities of the other type increased for 48 hours after pancreatectomy and then either returned to essentially the normal level or remained elevated. Serine dehydratase is an example of the former type.

Serine dehydratase induction, as mentioned earlier, can be prevented by glucose intubation by a mechanism independent of insulin secretion (Jost et al., 1967; Soling et al., 1969) and cyclic AMP (Sudilovsky et al., 1971).

Pituitary hormones seem to have no role in regulating serine dehydratase levels (Nakajima and Ishikawa, 1971). Nakajima and Ishikawa (1971) studied the activities of liver serine dehydratase and ornithine aminotransferase in hypophysectomised-pancreatectomised rats (3 days after the removal of pancreas) which were reduced to one tenth and one fourth respectively, of those in hypophysectomised rats. Liver histidase activity was also significantly decreased. This indicates that pituitary hormones including growth hormone play no significant role to depress these enzyme activities in pancreatectomised rats and supports the concept that those enzyme activities in rat liver are maintained by glucagon. In a previous report Ishikawa et al. (1965) had reported that serine dehydratase activity in rat liver is regulated by the antagonistic actions of insulin and glucocorticoids. However a gradual increase in the enzyme activity was observed

in the adrenalectomised-aloxan-treated rats, suggesting that there may be some hormone to enhance activity other that glucocorticoid. Although insulin inhibits the accumulation of cyclic AMP (Jefferson et al., 1968; Exton et al., 1972) and as such might be expected to inhibit the induction of serine dehydratse by glucagon, the available evidence suggests that this is not the case (Soling et al.,1969; Sudilovsky et al., 1971). Mohrenweiser and Yatvin (1972) showed that when amino acids are infused intravascularly, serine dehydratase is not induced in liver of adrenalectomised rats as it is when amino acids are given orally. The amino acids appear not to act directly on the liver to induce the enzyme. In fact, when injected intravascularly, they inhibit the normal glucagon induction of serine dehydratase, but not that induced by hydrocortisone. Jost et al., (1970) in their study of the early kinetics of serine dehydratase induction by cyclic AMP, glucagon and amino acids, suggested that cyclic AMP is an intermediate in the induction of the enzyme by glucagon and amino acids.

Dibutyryl cyclic AMP was as potent in inducing liver serine dehydratase as glucagon, and enhanced the enzyme activity twofold by itself and tenfold with concomitant injections of hydrocortisone acetate, which had no significant effect by itself, thus demonstrating a "permissive action" of glucocorticoid for the effect of dibutyryl cyclic AMP (Nakajima and Ishikawa, 1971). Several processes in liver cells known to be stimulated

by glucagon with the aid of the "permissive action" of glucocorticoid include urogenesis (Miller, 1961), gluconeogenesis (Friedman et al., 1967) and the induction of tyrosine aminotransferase (Greengard and Baker, 1966; C'sanyi et al., 1967) as well as serine dehydratase (Ishikawa and Nakajima, 1970). As reported earlier (Inoue and Pitot, 1970; Inoue et al., 1971) serine dehydratase has two distinct forms (isozymes) one of which is induced by glucagon and the other by glucocorticoid. It may well be expected that the form of serine dehydratase which is induced by glucagon responds more strongly to a combined administration of glucagon and glucocorticoid than to glucagon alone.

In adult adrenalectomised rats, fasting or glucagon administration increases its activity (C'sanyi and Greengard, 1967) and glucose prevents this increase (Goswani and Chatagner, 1966). Glucagon is part of the manifold equipment that regulates gluconeogenesis. But initiation of the development of enzymes involved in carbohydrate metabolism vital at birth may be one of the most important physiological functions of glucagon (Greengard and Dewey, 1967).

A study of the interrelationships of the action of dietary proteins and hydrocortisone on adaptive rat liver enzymes has shown that serine dehydratase was the only enzyme which did not respond to cortisone in the absence of dietary protein (Hurvitz and Freedland, 1968; Pitot et al., 1965). In the presence of 90% dietary casein, serine dehydratase activity

was enhanced by hydrocortisone due to an increased rate of its synthesis (Szepesi and Freedland, 1969).

As stated earlier, on administration of hydrocortisone acetate serine dehydratase was induced significantly in the livers of alloxan diabetic adrenalectomised rats, whereas little induction occurred in the livers of adrenalectomised rats with intact islets of Langerhans (Ishikawa et al., 1965). Freedland (1964) reported that serine dehydratase activity was enhanced by the administration of thyroxine. Preliminary data obtained in the laboratory of Ishikawa et al. (1965) showed that adminstration of triiodothyronine for five days caused some increase of serine dehydratase activity in adrenalectomised rats.

Fallon and Byrne (1967) found that no significant changes in lactic dehydrogenase or L-α-glycerophosphate dehydrogenase occur when cortisone acetate is adminstered for seven days to rats fed Purina Laboratory food.

Inhibitor studies utilising actinomycin D (Soling et al., 1969; Pitot et al., 1965), 5-fluoroorotic acid (Pitot and Peraino, 1964; Cihak et al., 1971) and 5-azacytidine (Cihak et al., 1971) indicated that the induction of serine dehydratase was dependant upon nucleic acid synthesis. Direct measurements of enzyme protein synthesis using immunological techniques showed that the increased enzyme levels after hormone or amino acid administration are the results of an increased rate of de novo enzyme synthesis

(Jost et al., 1968; Jost et al., 1970).

It is generally accepted that actinomycin D inhibits DNA-dependent RNA synthesis (Goldberg and Rabinowitz, 1962; Revel et al., 1964). It has also been reported that it interferes with the passage of newly formed RNA from the nucleus to the cytoplasm (Georgiev et al., 1963; Georgiev, 1967; Girard et al., 1964). Actinomycin D inhibited the induction of serine dehydratase promoted by dibutyryl cyclic AMP, suggesting that the cyclic nucleotide exerted its effect either at the level of transcription or transfer of the message to the cytoplasm. Actinomycin D prevents the induction of many enzymes by cortisone (Weber et al., 1965).

The concomitant administration of 5-fluoroorotic acid results in almost complete inhibition of the glucagon induction of serine dehydratase activity. This inhibition was enhanced by pre-treatment with glucosamine and galactosamine, probably through depletion of the intracellular uridine pools (Mohrenweiser and Pitot, 1974). Although less than a doubling of enzyme activity was observed after glucagon plus 5-fluoroorotic acid administration, 5-fluoroorotic acid affects RNA synthesis in mammalian systems. In contrast to actinomycin D, which blocks RNA synthesis, 5-fluoroorotic acid after conversion to 5-fluorouridylate is extensively incorporated into RNA by rat liver (Wagner and Heidelberger, 1962; Stenram and Willen, 1967). Preliminary data had suggested the existance of an enzymatically inactive "serine dehydratase-like" protein after 5-fluoroorotic

acid administration to rats (Pitot et al., 1968). Quantitation of a protein produced under these conditions with immunological properties similar to serine dehydratase indicates that after glucagon administration, 5-fluoroorotic acid has only a slight inhibitory effect on the accumulation of this "serine dehydratase-like" protein compared to the amount of native enzyme in glucagontreated rats with no analog. As 5-fluoroorotic acid is rapidly incorporated into cytoplasmic tRNA and mRNA (Wilkinson et al., 1971; Stenram et al., 1972), the inactive enzyme protein may be the product of translation of a mRNA containing 5-fluorouridine.

Fallon et al. (1966) studied the inhibition or activation of serine dehydratase by various additions in vitro. Glycine, ATP, ADP and AMP were without effect on serine dehydratase from rat liver. However, a marked inhibition by cysteine was apparent, confirming the observations of Kato et al. (1964).

Bond (1976) compared the stabilities of nine rat liver cytosol enzymes at a variety of pH values. The enzymes studied were those (a) with half-lives in vivo of 3 days or longer e.g. lactic dehydrogenase, arginase etc. (b) those with half-lives in vivo shorter than two days e.g. glucokinase, L-serine dehydratase and tyrosine aminotransferase and (c) catalase, which has an intermediate half-life of 2-5 days for the protein portion. She found that the enzymes were stable in vitro at neutral and alkaline pH values. However, at acidic pH values (pH 4.0) the long-lived enzymes (a) were stable, the short-lived enzymes (b) were completely

inactivated with one exeption and catalase (c) was partially inactivated. This property of acid instability may be of great significance *in vivo* in determining the functional half-lives of enzymes in terms of the irreversible loss of enzyme activity and suggest that lysosomes are involved in the initial stages of inactivation and degradation of intracellular protein. Acidic conditions are present in lysosomes (Reigngoud and Tager, 1973) and there is evidence indicating that lysosomes are involved in intracellular protein digestion (Dean, 1975). Therefore, Bond (1976) proposed that inactivation *in vitro* in acidic solution is correlated with activation *in vivo* or functional half-lives measured *in vivo*. One physiological implication of this finding is that the short-lived enzymes, e.g. L-serine dehydratase, may tend to denature in acidic portions of the cell and that unfolding may result in precipitation, adhering to membranes, vacuolization or sequestering of denatured proteins and perhaps subsequent digestion.

Thorndike (1972, 1973) found that serine dehydratase activity in adult liver is more than three times higher in mice than in rats, in agreement with the major species differences in enzyme activity found elsewhere (Nagabhushanam and Greenberg, 1965; Rowsell *et al.*, 1965). Hence it appears to be generally related to body size, e.g. to basal metabolism (Rowsell *et al.*, 1973).

Davis *et al.* (1970) studied the levels of serine dehydratase in Morris hepatoma cells. No correlation between tumour growth rate and serine

dehydratase was demonstrated. The serine dehydratase activity of the hepatomas did not respond to changes in protein intake in the same manner as the normal and host livers.

4. Serine dehydratase in foetal and neonatal animals

The serine dehydratase activity of foetal rat liver is very low; it normally begins to increase after birth, but the injection of glucagon to foetuses causes it to accumulate before birth (Ishikawa and Nakajima, 1970). Serine dehydratase (unlike tyrosine amino transferase) is not inducible by hydrocortisone in four-day old rats (Ishikawa and Nakajima, 1970). Muira and Nakagawa (1970) found that the serine dehydratase level was low in prenatal liver and was increased when glucagon was administered intraperitoneally to the foetus. Inductions due to glucagon was due to the increase in the rate of synthesis of enzyme molecules. Insulin repressed prenatal enzyme induction by glucagon and natural post natal development of liver serine dehydratase.

5. The organ culture of adult rat liver

Studies on enzyme regulation are greatly facilitated by the ease of a system for the maintenance of viable liver tissues in vitro. In such a system direct effects of hormones and other enzyme inducers on liver tissue can be studied. Liver tissue from one animal can be used in experiments that would normally require several animals to satisfy statistical requirements. In turn, for the maintenance of animal tissue in vitro

certain requirements have to be met; the tissue has to be supported, it must have access to a suitable nutrient supply and must be maintained within narrow limits of pH, temperature and osmotic potential (Willmer, 1965). To this end, earlier workers used natural media extensively both for nutrient supply and support. These included plasma clots (Fell, 1965) and agar (Wolf and Haffen, 1952) for support with various sera for nutrient supply. Later workers started using more synthetic materials, e.g. Chen (1954), who floated foetal organ cultures on tea bag paper, and Trowell (1959) who managed to maintain viability of several kinds of adult tissue supported on stainless steel grids and using a completely "synthetic" medium.

Both Fell and Trowell had considerable difficulty in culturing liver in organ culture. Epithelial cell cultures derived from liver tissue with some of the properties of liver tissue have been known for some time (Chang, 1954), but disaggregated tissues have been known to undergo dedifferentiation in long-term cell culture (Willmer, 1965). Several attempts at culturing primate liver in organ culture have been made. For example, Ingram (1962) maintained 1.5 to 2 mm liver cubes from Rhesus monkeys in roller tubes or Leighton tubes for periods of up to two days. He found media consisting of more than 20% foetal calf serum with 10% chick embryo extract not suitable. Lurie et al. (1969) and Laufs and Walker (1970) also experimented with primate liver but found the concentrated serum media necessary. The latter group emphasised the importance of oxygen in the culture atmosphere and demonstrated a differential incoproration of

^{3}H-leucine and ^{3}H-uridine into cytoplasmic and nuclear regions of the cells respectively by autoradiography.

Like Laufs and Walker (1970) several other workers (Ingram, 1962; Trowell, 1959; MacDougall, 1963) also emphasised the importance of oxygenation if cells in organ culture are to remain viable. Gerard (1931) derived a formula which predicts the expected thickness of surviving tissue in an organ culture;

$$C_o = Ar^2/6D + C_1$$

where C_o = oxygen tension at the surface of the tissue

C_1 = oxygen tension at the bottom of the tissue

A = oxygen uptake

D = diffusion coefficient

r = thickness of viability

The value given to C_1 is the minimum oxygen tension at which liver cells are able to survive. In a gas phase of 95% air and 5% carbon dioxide the majority of the cells were necrotic within 24-48 hours (Campbell and Hales, 1971). The primary cause of cell death appeared to be lack of oxygen. This results in a drop in the intracellular ATP concentration and hence loss of sodium pump activity. Measurement of the ATP and ion content shows that the pieces of liver are fully oxygenated by 3 atm. O_2. Under these conditions within 24 hours toxic effects of oxygen are apparent, making it impossible to maintain a viable culture in 3 atm. oxygen. Laufs and Walker (1970) found that in primate liver organ culture, 60% oxygen resulted in the largest number of viable cells and that 95% oxygen was toxic.

They also published a method for adult rat liver where the liver was cut to cubes 1,5 mm square and 0,5 mm thick and placed on grids scratched on Petri dishes. They added 1.5 ml of medium (Basal Eagle Medium) and the cultures were kept under an atmosphere consisting of 95% oxygen and 5% carbon dioxide. A zone of cells at the gas-tissue interface appeared to remain viable for at least 6 days and formed a viable zone for tissue 0,25 to 0,30 mm thick. Like the 65% oxygen condition with primate liver they had prominant nucleoli. Their results showed that the pieces of liver contained an appreciable amount of ATP which remained fairly constant between 1 and 6 days. The ion content also remained fairly constant over this period. Although there was a loss of potassium from the pieces during the first 24 hours in culture they still retained 70% of the potassium content of normal liver. Siddle et al. (1973) subsequently demonstrated that glucagon was able to induce cyclic AMP levels in these cultures and Campbell (1973) was able to induce tyrosine aminotransferase with dexamethasone.

Recently methods have appeared for the culture of non-dividing parenchymal adult rat liv : cells (Michalopoulos and Pitot, 1975; Pariza et al., 1975). These are based on the method of dispersing the cells from the organ using collagenase according to the method of Berry and Friend (1969) and allowing them to attach to a collagen support. The cells thus attached are maintained in essentially synthetic media, supplemented with insulin and glucose, and are able to remain viable for periods up to 20 days. The criteria of viability include the uptake of ^{3}H-leucine and ^{3}H-cytidine as

demonstrated by autoradiography as well as the appearence of the cells under light and electron microscopy.

Wicks (1968) in his studies on organ culture in foetal rat liver used a gas phase of 98% air and 2% carbon dioxide and found that the histological appearance of the hepatocyte nuclei was unchanged - although there was some loss of cytoplasmic vacuolization over a 52 hour period in culture, perhaps due to a loss of glycogen.

Wicks (1968) also found that lactic dehydrogenase activity in foetal rat liver organ culture was not altered after a 17 hour exposure to hydrocortisone at the same time that tyrosine transaminase activity increased fivefold. It is known that only a limited number of hepatic enzymes are influenced by glucocorticoids (Rosen and Nichol, 1963). The fact that the hormone has no detectable effect on protein synthesis or on the activity of another enzyme in organ culture suggests that the response of foetal livers to glucocorticoids is as selective as that in adult liver. Streffer and Williamson (1965) found that slices of rat liver immersed in nutrient media for 1 or 2 hours release a considerable portion of their protein content. MacDonnel et al. (1975) in their study of liver explants from 20 day old foetuses cultured for 48 hours in the absence of serum, released 70% of their total suluble protein content in the medium. In the presence of serum this loss still amounted to 60%. The addition of cortisol plus glucagon (to serum containing media) did not decrease the loss of total soluble protein from the explants but induced considerable tyrosine

aminotransferase activity which was not released into the medium, while lactic dehydrogenase (E.C.1.1.1.27) was released into the medium.

6. Purpose of Research

The aim of this research was to study the control of the enzyme serine dehydratase (E.C.4.2.1.13) under *in vitro* conditions. In spite of considerable literature on the control of serine dehydratase *in vivo* no studies have been done on this enzyme under the more readily controlled conditions of organ culture of adult rat liver.

A study of the enzyme levels of serine dehydratase, both *in vivo* and *in vitro* using two different assay methods and under different hormonal and dietary conditions was undertaken. Also a study of the activity of another soluble enzyme, lactic dehydrogenase, was undertaken and the *in vivo* and *in vitro* levels were compared under conditions comparable to those used with serine dehydratase.

An attempt was also made to compare the activity of serine dehydratase in 13-day old to that of adult rats *in vitro*.

Because of the results found, attention has been given to the histological state of the liver and slices and its potassium ion content.

II MATERIALS AND METHODS

1. Chemicals

L-Serine, pyridoxal phosphate, trichloroacetic acid (TCA), potassium phosphate buffers, 2,4-dinitrophenylhydrazine, sodium hydroxide, potassium chloride, tris-HCL buffers were from E. Merck, Darmstadt, Germany. Ethylenediaminetetraacetic acid (disodium salt) (EDTA), calcium gluconate, sodium bicarbonate, copper sulphate, sodium carbonate, potassium tartrate, 2-mercaptoethanol were from British Drug Houses Ltd., Poole, Dorset, England. Bovine serum albumin (BSA), dbc AMP (N^6,O^2-dibutyryl adenosine-3′:5′-cyclic monophosphoric acid), hydrocortisone-21-sodium succinate, dithiothreitol (DTT), 3,3′,5-triiodothyronine (T_3) and 3,3′, 5,5′- tetraiodo-L-thyronine (T_4) were from Sigma Chemical Co., St. Louis, U.S.A. Glucagon was from Lilly Laboratories, Short Street, Isando, Transvaal. Betsolon (betamethasone sodium phosphate), penicillin as crystapen (sodium benzylpenicillin B.P.) was from Glaxo Allenbury's, Wadeville, Transvaal. NADH was from Boehringer Mannhein, W. Germany, sodium pyruvate from L. Light and Co., Ltd., Colnbrook, U.K., concentrated hydrochloric acid by P.A.L. Chemicals. Medium M199 with Hanks Salts with L-glutamine and without sodium bicarbonate and egg ultrafiltrate were from Grand Island Biologicals, Grand Island, New York, U.S.A. Streptomycin as Novo Strep (Streptomycin sulphate B.P.) was from Novo Industries (Pharmaceuticals) (Pty) Ltd., 203 Main Street, Johannesburg, S.A. Foetal bovine serum microplasma (virus screened)

was from Flow Laboratories Ltd., Victoria Park Hesther House Road, Irvine, Scotland, and Thalamonal was from Janssen Pharmaceuticals, Belgium.

Chemicals for histological studies were obtained as follows :-formalin, millonigs buffer, and uranyl acetate from British Drug Houses, Glutaraldehyde and propylene oxide from Merck, osmium tetroxide and toliudine blue from Emscope, 13 Bedford Road, London; epon, araldite and lead citrate from Polaron Equipment Ltd., Watford, England, haematoxylin and distrenedibutylphthlac-xylol (dpx) from Ehrlichs Chemicals and alcohols, benzene, xylene, paraffin wax and ethanol from central stores, Wits University Johannesburg.

2. Animals

Animals were supplied either by the Central Animal Unit, Frankenwald, Johannesburg, or by the Department of Pharmacology, University of the Witwatersrand, Johannesburg. Thirteen day old rats were bred in the Department of Biochemistry of the same University. Rats (Rattus norvegicus) used for in vivo and in vitro organ culture experiment were of the Sprague Dawley strain. Rats were obtained at 100-120 gm weight and allowed to acclimatise for a few days before the commencement of experiments. The animals were maintained four to a cage in standard laboratory cages (Labotec (Pty) Ltd., Johannesburg) and when needed were killed by cervical dislocation.

2.1 Feeding

All rats were fed ad Lib. on mice cubes containing approximately twenty per cent protein (Delmas Milling Company, Ltd., Johannesburg). For fasting regimens food was removed from the cages 24 hours prior to the commencement of experiments.

2.2 Adrenalectomy

Adrenalectomy was performed by a variation of the method of D'Amour and Blood (1965). All instruments and paper towels were sterilized by autoclaving before use. The rat to be operated on was anaesthetised with thalomonal (Janssens Pharmaceutica, Belgium), 0.15 ml per 100 g body weight, administered intramuscularly in one of the hind legs. The hair around the operation site (mid back) was shaved with a razor and the skin washed with soap and warm water. The rat was then placed on a sterile towel and the skin was cleaned with 70% alcohol. A dorsal mid-line incision was made from approximately the tenth thoracic to the third lumber vertebra and the skin was retracted laterally to the right using a fine curved artery clamp. A small hole was cut into the muscle wall just anterior to the upper pole of the kidney using a sharp pointed pair of scissors and enlarged slightly by retracting the scissors. The adrenal gland, embedded in fat and connective tissue, was slightly withdrawn from its position using long

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fairly blunt forceps and dissected with the sharp pointed scissors. No ligations of the blood vessels supplying the gland were necessary. The muscle wall was closed with one or two stitches. This process was repeated for the left side. The wound was finally stitched and sprayed with liquidoplast. Operated animals were placed in clean cages and allowed immediate access to food. The drinking water was supplemented with 0.9% saline.

2.3 Thyroidectomy

Thyroidectomy was performed by a slight variation of the method of D'Amour and Blood (1965). All instruments and paper towels were sterilized by autoclaving before use. The animal to be operated on was anaesthetised with thalamonal (0.15 ml per 100 gm body weight) administered intramuscularly in one of the hind legs. The hair around the operation site was removed and the skin washed thoroughly with warm water and 70% alcohol. A ventral mid-line incision through the skin of the neck was then made. The salivary glands were pushed to one side by slitting the overlying muscle in the midline and a sterilised retractor inserted. The prongs of the retractor were pushed apart in such a way that it exposed the trachea and the reddish, bi-lobed structure lying on either side of the trachea just below the larynx. The para-thyroids were seen as tiny whitish bodies at the tip of the upper lobe and an effort was made to leave them behind. The

isthmus connects the two lobes across the tracea. After dividing the isthmus using a pair of fine forceps and while holding on to one end, dissection was carefully made underneath the lobe. The inferior thyroid artery, which is the main blood supply to the thyroid and the recurrent laryngeal nerve, which lies close to the trachea and passes below the lobe of the thyroid, were avoided. The artery was clamped off with the tips of a fine hemostat, while the nerve was not touched at all as respiratory difficulties could develop later resulting in the death of the animal. The lobe was dissected free leaving a small amount at the upper tip enclosing the parathyroid, and removed. The other lobe was removed in a similar manner. The prongs of the retractor were slowly released and removed. The wound was closed with fine silk sutures through the muscle and two or three more through the subcutaneous tissue. The skin was stitched together with slightly thicker silk sutures and sprayed with liquidoplast.

Operated animals were placed in clean cages and allowed immediate access to food. The drinking water was supplemented with 0.9% saline and 1% calcium gluconate (to avoid parathyroid tetany). The animals were watched over the next few days for any signs of respiratory distress and kept for at least two weeks before being used for experiments.

2.4 Double operations - Thyroidectomy/Adrenalectomy

When animals were subjected to double operations they were first thyroidectomised. Adrenalectomy was performed a week before the commencement of experiments. The drinking water was supplemented with 0.9% saline, 1% calcium gluconate and 0.1% potassium chloride.

2.5 Sham operated rats

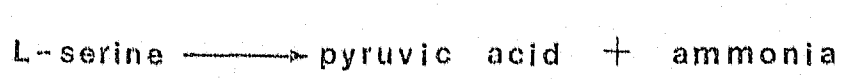
The animals were anaesthetised and kept in a prone position. The skin was washed and the hair removed. The skin was washed with 70% alcohol. A mid-line incision was made as if one was about to perform an adrenalectomy. The whole procedure for adrenalectomy was followed except that the adrenals were not removed. The wound was closed followed by stitching the skin together and spraying with liquidoplast. Similarly a sham operation was performed for thyroidectomy.

3.0 Enzyme Assays

Serine dehydratase was assayed by two methods.

3.1 Colorimetric Method

Serine dehydratase catalyses the reaction:



The pyruvate formed can be colorimetrically determined by conversion to the 2,4-dinitrophenylhydrazone. The method described here is a modification of the method of Friedman and Haugen (1943).

The livers from rats were weighed and quickly homogenised in 9 volumes of 0.05 M potassium phosphate buffer pH 7.2 containing 0.15 M potassium chloride, 0.001 M EDTA, 0.001 M 2-mercaptoethanol, 0.001 M DTT and 0.0005 M pyridoxal phosphate in a glass Potter homogeniser. The homogenate was centrifuged at approximately 27,000 x g in a Sorvall RC2-B centrifuge for 30 minutes. Activity in the resulting supernatant was assayed in a total volume of 1.0 ml containing 0.1 M potassium phosphate buffer pH 8.0, and 1 mM EDTA, 50 mM pyridoxal phosphate and 100 mM L-serine previously warmed to 37°C. The reaction was started by the addition of 50 µl enzyme extract and the reaction mixture further incubated for five minutes. The reaction was stopped by the addition of 0.5 ml of cold 10% TCA solution. Any precipitate formed was removed by centrifugation after the preparation had stood for 10 minutes in ice bath. A 0.5ml aliquot was pipetted into 0.5 ml of 2,4-dinitrophenydrazine (0.033% in 2N HCl) solution and was allowed to stand for at least 5 minutes at room temperature. Two ml of 2N sodium hydroxide solution was added to the aliquot mixture. The optical density was then read at 450 nm in a Hitachi model 101 spectrophotometer within 5 minutes after the addition of sodium hydroxide. For control tubes the TCA solution was

added prior to the addition of the enzyme extract. A standard curve was also done with each set of experiments. A 2 mM stock solution of sodium pyruvate was prepared and futher dilutions were made to give series of concentrations of 0.1 to 0.5 µmol pyruvate per ml (fig 1) Figure 2 shows the rate of pyruvate formation on incubation.

3.2 Enzymatic Method

Serine dehydratase was assayed at room temperature according to the method of Wimhurst and Manchester (1973) with slight modifications. The enzyme was extracted by homogenization of the liver in 9 volumes of 0.1 M K_2HPO_4, pH 8.6, containing 0.05 mM pyridoxal phosphate, in a glass homogeniser, specially designed to deal with small quantities. Activity in the supernatant, after centrifugation for 30 minutes at approximately 27,000 x g in a Sorvall RC2-B centrifuge was assayed in a total volume of 3.0 ml. The assay mixture contained 50 mM Tris-HCl buffer pH 8.6, 112 mM potassium chloride, 0.05 mM pyridoxal phosphate, 0.25 mM NADH and 300µl of enzyme extract. The reaction was started by the addition of 0.1 ml of a 3 M solution of serine, to give a final concentration of 100 mM . The rate of disappearance of NADH was followed with Pye Unicam SP 8-100 ultraviolet recording spectrophotometer at 340 nm. The rate of disappearance of NADH in the absence of serine was subtracted from that in its presence. A comparison between the two assay methods is shown in Table 1.

3.3 Lactic Dehydrogenase

Lactic dehydrogenase was assayed at room temperature by a method similar to that for serine dehydratase described in section 3.2. The enzyme was extracted by homogenization of the liver in 9 volumes of 0.1 M K_2HPO_4, pH 8.6, containing 0.05 mM pyridoxal phosphate in glass homogenisers specially designed to deal with small quantities. Activity in the supernatant, after centrifugation for 30 minutes at approximately 27,000 x g in a Sorvall RC2-B centrifuge, was assayed in a total volume of 3.0 ml containing 40 mM Tris-HCl buffer pH 8.6, 90 mM KCl, 0.05 mM pyridoxal phosphate, 0.25 mM NADH and 50 µl enzyme extract. The reaction was started by the addition of 20 µl of a 1mM solution of sodium pyruvate to give a final concentration of 0.0066 mM. The rate of loss of NADH was followed with Pye Unicam SP8-100 ultraviolet recording spectrophotometer at 340 nm. The rate of disappearance of NADH in the absence of sodium pyruvate was subtracted from the value obtained in its presence.

3.4 Protein Estimation (Lowry)

Protein was determined by the method of Lowry *et al.* (1951). In brief the method consists of :

Reagents

A 2% Na_2SO_3 in 0.4% NaOH

B 1% $CuSO_4$

C 1% potassium tartrate

D 0.5% bovine serum albumin (5 mg/ml)

E Folin Reagent, diluted 1:2 with distilled water

Procedure

50 parts A, one part of B and one part of C were mixed together. To 5 ml of this mixture was added 1 ml of a suitably diluted protein sample, mixed and left for 5 minutes. To this was added 0.5 ml of diluted Folin reagent (E), mixed and left for 30 minutes and read at 660 nm in a Hitachi model 101 spectrophotometer.

For a standard curve a range between 40-300 mg BSA per ml was added (Fig. 3)

3.5 Calculations

Activities of enzymes are expressed as follows:-

Serine dehydratase - µmol pyruvate or NAD produced per min per gram liver.

When the colorimetric assay method was used the enzyme activity was

expressed as µmol pyruvate produced per min per gram of liver and when the enzymatic assay method was used the activity was expressed as µmol NAD produced per min per gram of liver.

Lactic dehydrogenase - µmol NAD produced per min per gram of liver.

For the colorimetric assay of serine dehydratase a standard curve relating colour production to pyruvate added is shown in Fig. 1. Fig. 2 shows the effect of incubation time on pyruvate production. A linear relationship was observed. An incubation time of 5 minutes was routinely used in most experiments.

Fig. 3 shows a standard curve for protein estimation.

Table 1 gives comparison of serine dehydratase activity determined by the colorimetric and enzymatic methods. No significant differences are seen suggesting that the colorimetric assay is satisfactory.

3.6 Statistical Evaluation of Results

The mean of values of a group of assays was calculated from the formula :

$$\bar{x} = \frac{\Sigma x}{n}$$

where $\bar{x}$ signifies the mean value of x, where x = individual results and n = number of determinations.

FIG. 1

Standard curve relating absorbance against amount of pyruvate in the colorimetric assay

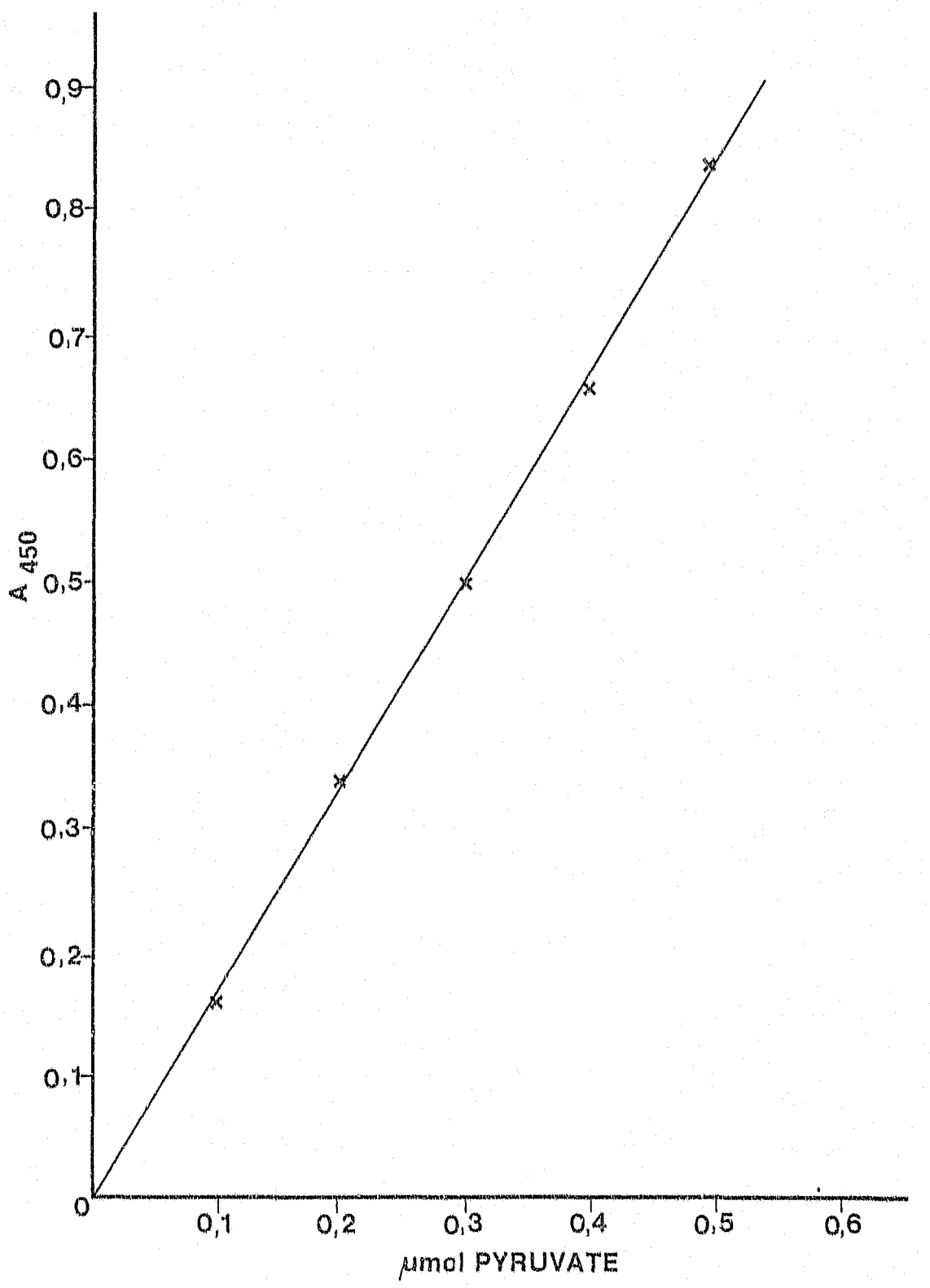


FIG. 2

Effect of incubation time of the assay of serine dehydratase (total product formed)

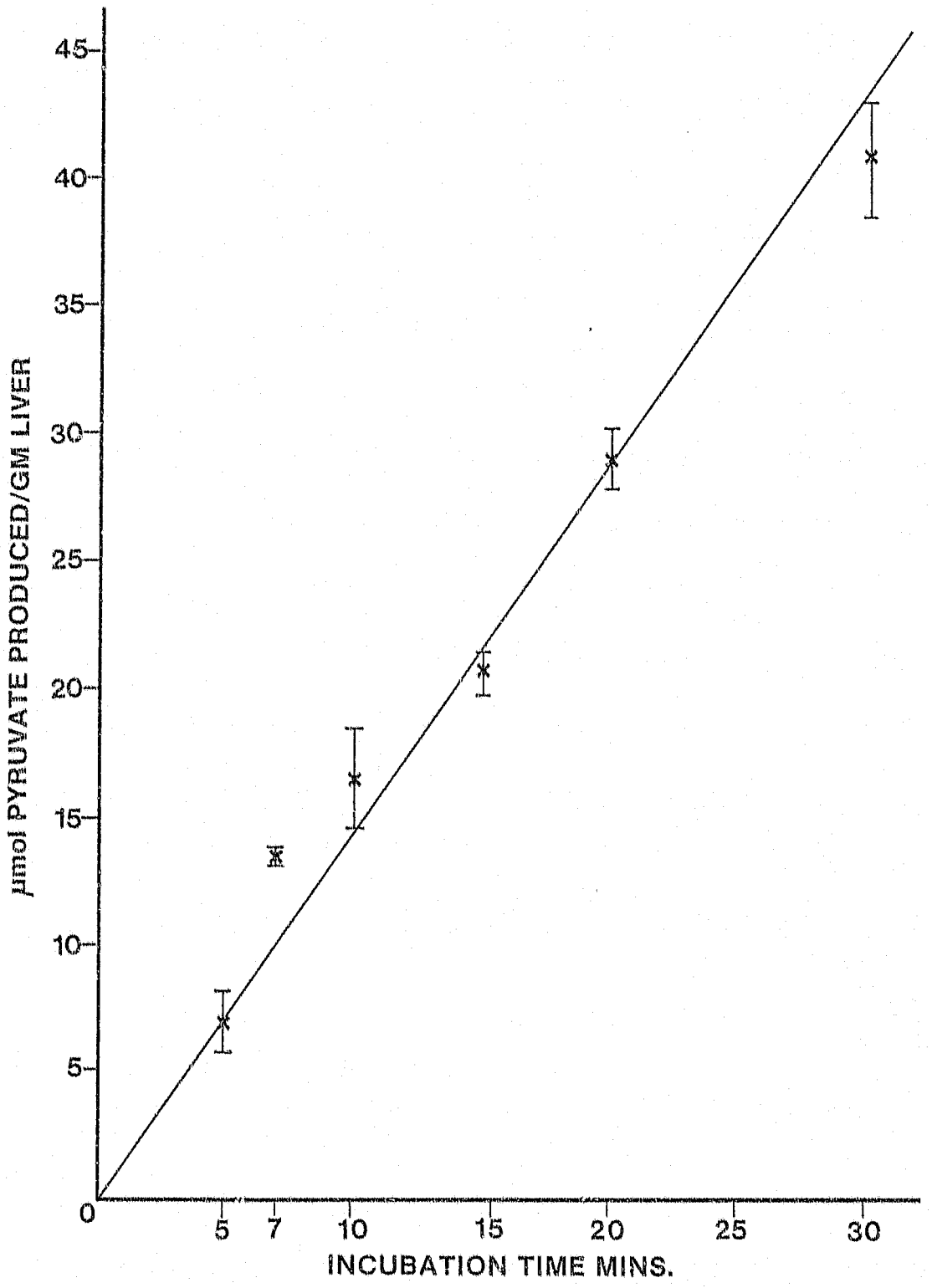


FIG. 3

A protein calibration curve relating absorbance against protein concentration measured by the procedure of Lowry *et al.* (1951)

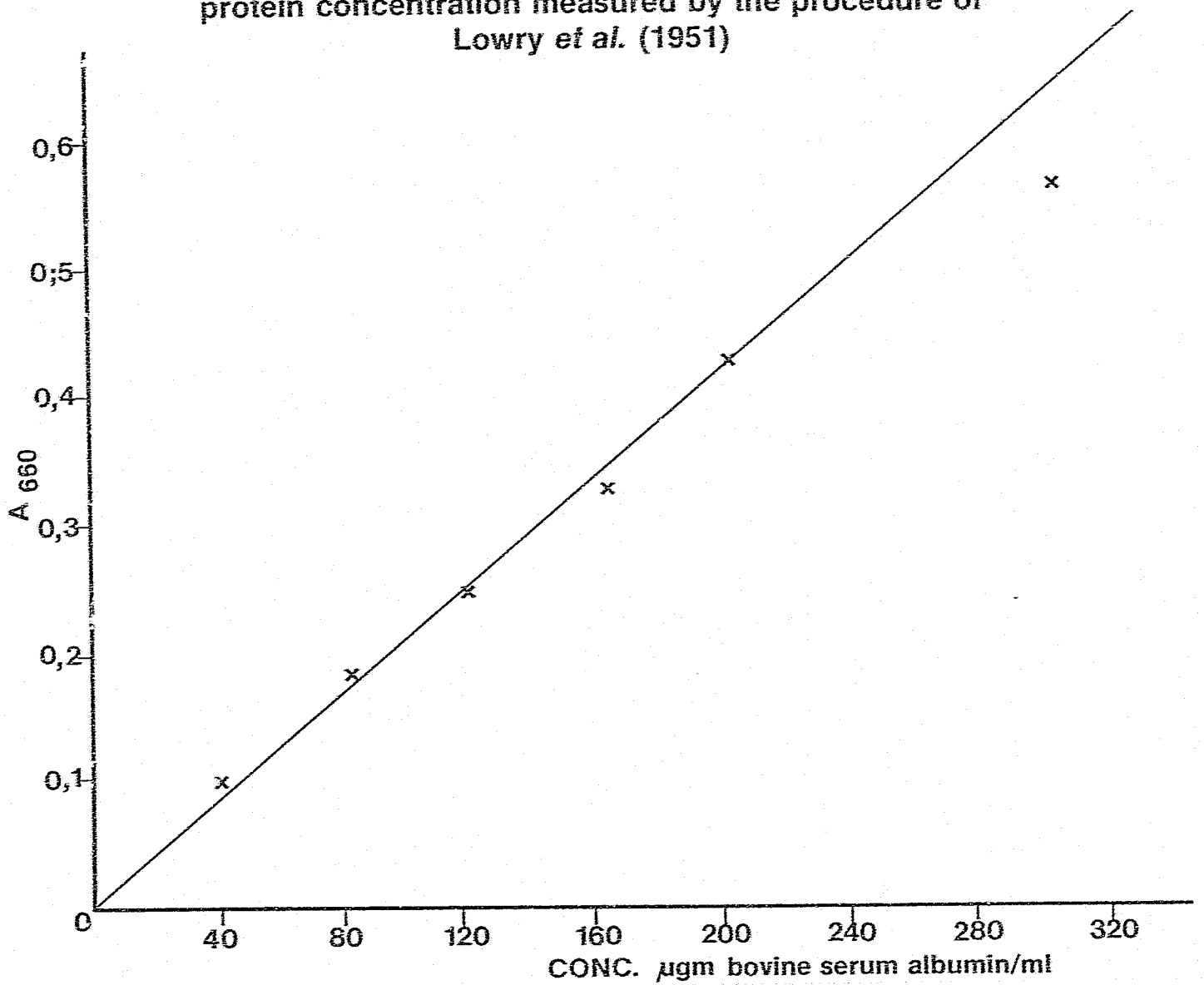


TABLE 1

Comparison of two different assay methods on the measured activities of serine dehydratase in fresh liver

Colorimetric assay (μmol pyruvate produced per min/gm liver)	1.90 ± 0.10
Enzymatic assay (μmol NADH used per min/gm liver)	2.31 ± 0.48

The details of the two assays are as described in section 3 of Materials and Methods.

All mean values are quoted with standard errors ($\pm$ S.E.M.). The standard error of the mean is given by the formula :-

$$\sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$$

Limits of significance were determined by the t-test using the formula :

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

where $\bar{x}_1$ = mean value of one set of determinations and $\bar{x}_2$ = mean value of second set of determinations, SE_1 = standard error of x_1, SE_2 = standard error of x_2.

When comparing the effects of two alternative treatments or experiments it is sometimes possible to make comparisons in pairs. This is called the paired t-test and is calculated by the formula :

$$\bar{d} \ / \ (\text{SE of } d)$$

where $\bar{d}$ = the average difference between the number of pairs.

Values of t greater than those for differences with ninety five per cent confidence were regarded as significant.

4. Organ Culture of Adult Rat Liver

4.1 Preparation of apparatus

A standard cleaning procedure was followed for apparatus that came

into contact with the culture medium or the tissue. Glassware and dissecting instruments were first scrubbed using normal laboratory detergent, rinsed with tap water and soaked in a 1% contrad solution (Hickman and Kleber, Durban). This was followed by another rinse in tap water, and two rinses in distilled water. No cleaning procedure was followed for filter paper. The apparatus was sterilised by autoclaving for 20 minutes. Dissecting instruments were sterilised in OK sterilisation bags (Propper Manufacturing Co., New York), beakers were covered with aluminium foil, filter paper was kept in Petri dishes, flasks and measuring cylinders were stoppered with cotton wool and culture dishes (No. 1 Conway Units, Gallenkamp, England) were covered with lids autoclaved as such.

4.2 Medium

The method of preparation of medium and procedure was according to the technique of Mattheyse (1977) with modifications. The medium consisted of 50% bovine serum, 10% whole egg ultrafiltrate and 40% M199 with Hanks balanced salt solution containing sodium bicarbonate, penicillin and streptomycin as antibiotics.

The whole egg ultrafiltrate was prepared by mixing Grade A large eggs with saline, and pressure filtering through a membrane of 15Å pore size. This excludes materials of molecular weight greater than 12 000. The

ultrafiltrate is protein free, as tested with 10% TCA.

M199 with Hanks' balanced salt solution (Morgan et al., 1950, and Hanks and Wallace, 1949) with L-glutamine and without sodium bicarbonate was supplied by Grand Island Biological Co. in powder form. The powder came in packets, each packet containing enough powder for 1 litre of medium. The contents of a single packet were dissolved in about 500 ml distilled water, which contained 0.35 gm sodium bicarbonate, 30 mg penicillin as crystapen and 50 mg streptomycin as Novostrep. The contents were mixed and the volume made up to one litre. The pH at this stage was about 6.8, but after pressure filtering through a Millipore filter membrane (Millipore Corporation, Bedford, Massachusetts, U.S.A.) packed with filters having pore sizes at 0.22μ , 0.45μ , and 1.2μ , interspaced with a layer of dacron, plus a prefilter, it soon adjusted to between 7.2 and 7.3 and was stored at 4^{o}C in 250 ml quantities.

Bovine serum was prepared from blood obtained at the local municipal abattoir. It was collected septically in a clean 10 litre beaker and allowed to coagulate at room temperature for about three hours. After twenty four hours in the cold room the clot formed was lightly broken with a glass rod and the serum poured off and centrifuged at about 20,000 x g for 30 minutes in Sorvall RC2-B centrifuge to remove blood

cells and minute clot material. The supernatant was collected and pressure-filtered through a Millipore filter membrane (previously sterilised) and stored frozen in 100 ml quantities. Just before use, the serum was thawed and heated to 55°C for 20 minutes to inactivate complement.

Any additions to the medium such as hormones were dissolved septically in the synthetic component of the medium (M199), the quantity measured out to give the desired final concentration in the composite medium. This was then sterilised through a Millipore swinnex filter, fitter with 0.22 μ membrane. The medium components were dispensed on a special sterile bench (Fibratron pure work station, S.A.), using sterile measuring cylinders, into 250 ml Ehrlenmeyer flasks and gently swirled around. It was then decanted into the centre wells of the culture vessels and covered with sterile Whatman's No. 1 filter paper ensuring that no air bubbles were trapped.

4.3 Culture procedure

The animal s killed and the liver removed outside the sterile chamber. After killing, the operator's hands and the rats abdomen were swabbed with 70% alcohol. A ventral mid-line incision using sterile scissors was made into the skin from about mid-thorax to the abdomen. Lateral incisions were made at either end of the cut and the flaps of the skin were folded back. The muscle wall was then swabbed with 70% alcohol

and the incision was repeated into the peritoneal wall. The xiphisternum was removed. The various lobes of the liver were carefully dissected free from falciform ligament, hepatic portal veins and bile canals and put into about 50 ml sterile synthetic medium in a beaker covered with foil. This was transferred to the sterile room where the operator's hands were thoroughly washed with Gill soap and a face mask worn. The liver lobes were washed free of excess blood by swirling them in the beaker. They were then transferred to clean sterile synthetic medium in a second 100 ml beaker.

A lobe of the liver was removed from the beaker and dried on a filter paper. It was then transferred to another filter paper and spread out. The sides of the liver were cut to give a rectangular cutting face. Sterility was slightly waived at the cutting stage when the liver was clasped in some filter paper with the thumb and forefinger of the one hand. Culture slices were sectionised from the 'cutting face' using a surgical skin-graft knife or dermatome (Downs Bros., and Mayer and Phelps Ltd., Surrey, U.K.) set to 0.75 mm. Cutting was best done by using a sawing motion. Sections cut to about 0.3 mm thick were collected from the edge of the knife and floated in a petri dish with some synthetic medium. When enough tissue was collected the slices were swirled gently and transferred to the culture dishes with sterile forceps where they were spread out to about two or three slices per dish to ensure maximum contact with the medium and gas phase.

The cultures (Fig. 4) were placed in a specially designed box made of 112 mm thick transparent perspex (Fig. 5). The box was designed to hold a maximum of three sets of cultures, each set consisting of four Conway units taped together one above the other. The cultures rested on a loose platform with perforations. The gas entered from under this platform and left from just under the lid which was screwed on to make the box air tight. The cultures were then perfused with 95% oxygen and 5% carbon dioxide for 10-15 minutes. The inlet and outlet valves were then closed and the box put in an incubator set at 37°C. After 12 hours the atmosphere was renewed.

The pH of the medium usually dropped to about 6.8 but this was due to the high carbon dioxide atmosphere, since culture medium at this pH left standing in a normal atmosphere regained a pH of about 7.2. Where the medium was to be 'changed', culture dishes with the new medium were incubated simultaneously with the cultures from the onset, and at the appropriate time, the pieces of filter paper with the culture slices were transferred *in toto* to the new culture dishes. When hormones or any other additions were to be made to the medium, the medium mixtures were prepared just before the cultured slices were transferred.

The cultures were kept for 24 hours to 48 hours. When they were kept for 48 hours the atmosphere was renewed after 24 hours. In some experiments the cultures were kept for a futher 8-10 hours after 48

FIG. 4 Diagram depicting the culture system

a) Culture vessel, No. 1 Conway Unit, with ground glass lid.

b) Medium mixture

c) Whatman No. 1 filter paper disc.

d) Liver, cut to less than 0,75 mm thick.

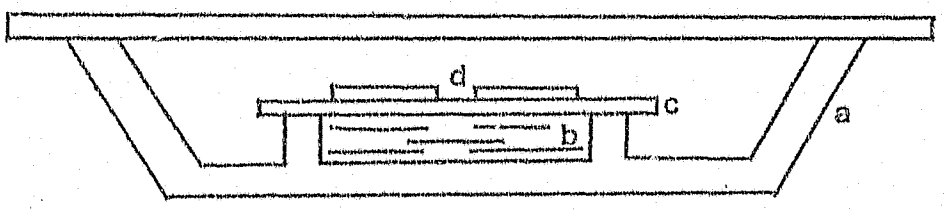


Fig. 5 Special Incubation Box

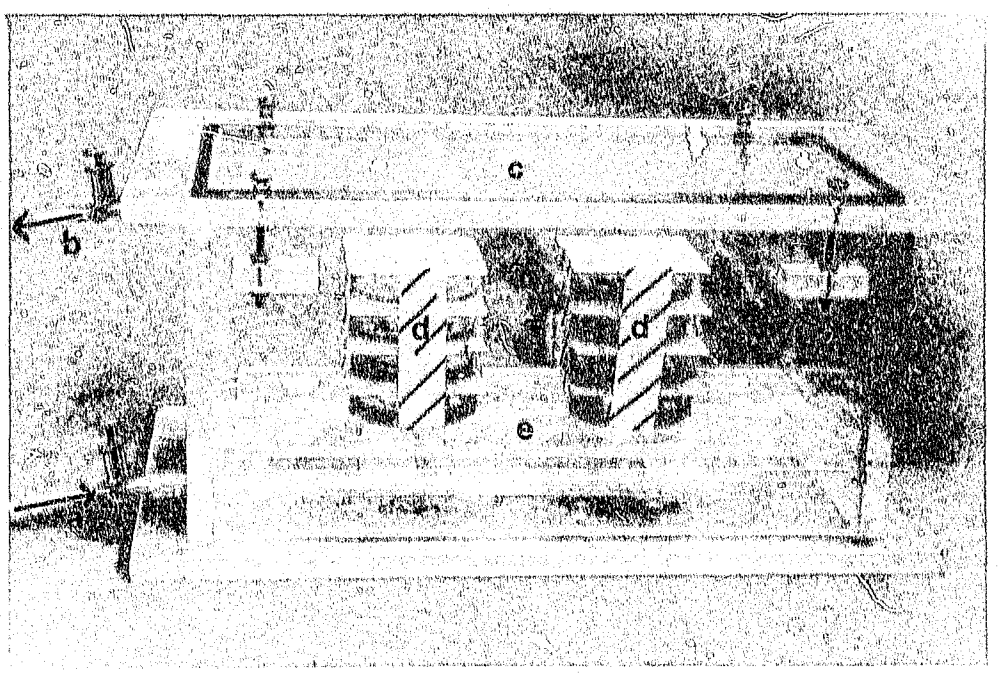
a) Inlet for gas mixture

b) Outlet for gas mixture

c) Screw on top

d) Sets of cultures

e) Perforated tray on which sets of cultures are placed



hours. (Section 2.6.1).

5. Organ Culture of liver of 13-day old rats

5.1 Preparation of apparatus

The apparatus is the same as for adult organ culture.

5.2 Medium

Instead of bovine serum from the abattoir, foetal bovine serum (FBS), microplasma and virus screened (Flow Laboratories, Scotland), containing whole egg ultrafiltrate was used. The synthetic M199 with Hanks balanced salts was the same as in adult organ culture. The medium mixture consisted of 50% FBS and 50% M199 with Hanks' balanced salts cotaining sodium bicarbonate, penicillin and streptomycin. The rest of the procedure was the same as with adult organ culture.

5.3 Culture procedure

Because of the size of the animal one had to be very careful in removing the liver. The rats were killed and the skin cut laterally across the abdomen and slowly removed by hand. The muscle was washed with 70% alcohol and incisions were repeated into the peritoneal wall. The xiphisternum was very carefully removed. Very slowly with a fine pair of scissors and forceps the lobes of the liver were freed from falciform ligaments, the hepatic portal veins and the bile canals. The lobes were

carefully transferred with a pair of blunt forceps into a beaker containing 50 ml of sterile synthetic medium covered with foil. In the sterile room they were transferred to another beaker containing synthetic medium. The lobe was dried on a filter paper and then slices cut with a dermatome with screws adjusted to 0.75 mm. The slices were cut by vertical motions of the knife across the surface of the liver lobe. The rest of the procedure was the same as in the adult rat.

Slices were cultured for 12, 24 and 48 hours. Also a further incubation of 8-10 hours after the initial 48 hours incubation was done. In a 24 hours incubation the atmosphere was renewed after 12 hours at the same time as cultures were transferred to new medium. For 48 hours the atmosphere was renewed after 24 hours. The gas used was a mixture of 95% oxygen and 5% carbon dioxide and the units were incubated at 37°C in an incubator.

6. Histological Techniques

6.1 Light Microscopy

Tissue for light microscopic study was fixed in buffered formalin at neutral pH and sections were prepared by Mr A Wadee of the Department of Immunology, S.A.I.M.R., Johannesburg. The fixed tissue was dehydrated by passing it through an alcohol gradient, cleared with benzene and embedded in paraffin wax in block form. The blocks were put on ice

to cool and then trimmed and later cut with a rotary microtome (5 microns). The tissue was picked up onto a microtome knife and then put on a glass slide. The slices were floated on a water bath, picked up and put on a glass slide, labelled, dried and placed on a hot plate for 30 minutes. The tissue was dewaxed with xylene, hydrated by passing through an alcohol gradient, washed in water and then dipped in Scotts tap water. The tissue was stained by adding haematoxylin (10 minutes) and then washed in tap water and dipped in eosin (2 minutes). It was then dehydrated by passing through an alcohol graaient put in xylene and mounted on distrene-dibutylphthalac-xylol (dpx). A cover slip was put on and the slide dried and cleaned. The tissue was viewed on a Reichert Univer photomicroscope belonging to the Dental Research Unit, University of the Witwatersrand, Johannesburg. A green filter was used for contrast while photographing various sections of the stained tissue.

6.2 Electron Microscopy (Kay (ed) 1965)

Tissue for electron microscopic study was fixed in 3% glutaraldehyde-formalin solution buffered in Millonigs phosphate (pH 7.4) buffer. The fixed tissue was washed three times in Millonigs phosphate buffer (pH 7.4) and then post fixed in 1% osmium tetroxide in Millonigs buffer. Sections prepared with the help of Miss Anna Botes of the Department of Electron Microscopy, S.A.I.M.R. Johannesburg. After washing with 50% ethanol, the tissue was passed through an alcohol gradient, cleared

with propylene oxide and embedded in an epon-araldite mixture and left overnight to polymerize at 80°C in an oven. Thick sections (1 micron) were cut with an LKB ultramicrotome and stained with toluidine blue. The sections were further trimmed to smaller block size and thin sections (500 - 700Å) were cut onto a glass knife filled with distilled water. The sections were picked up onto a copper grid and the grid was stained with uranyl acetate in 50% alcohol (10 minutes). The stained grid was rinsed three times in 50% ethanol, blotted and then put onto a drop of lead citrate for 10 minutes (Reynolds, 1963), and covered with a beaker (to avoid lead oxide formation). The stained tissue was rinsed three times in double distilled water, dried on a filter paper and then viewed. Viewing was done on an AEI 801 transmission electron microscope and negatives prepared.

7. Estimation of Potassium in Liver Slices

A simple procedure was used to estimate the potassium levels in rat liver slices. The rats were killed, livers removed and slices prepared and incubated as mentioned in Materials and Methods (4.3).

After incubating the slices, they were removed and weighed in a test tube. Two ml of distilled water was added to the slice and the tubes placed in a boiling water bath for 10 minutes. The supernatant was measured for potassium in an EEL flame photometer (Evans Electroselenium Ltd., Holstead, Essec, England), containing a potassium filter. The

instrument was calibrated to zero with distilled water and a 100% response with the most concentrated standard potassium chloride solution (1.5 mM). A calibration curve was plotted using 0.5, 1.0 and 1.5 mM standard potassium chloride solution (Fig. 6). After each estimation, the flame photometer was flushed with a sample of distilled water.

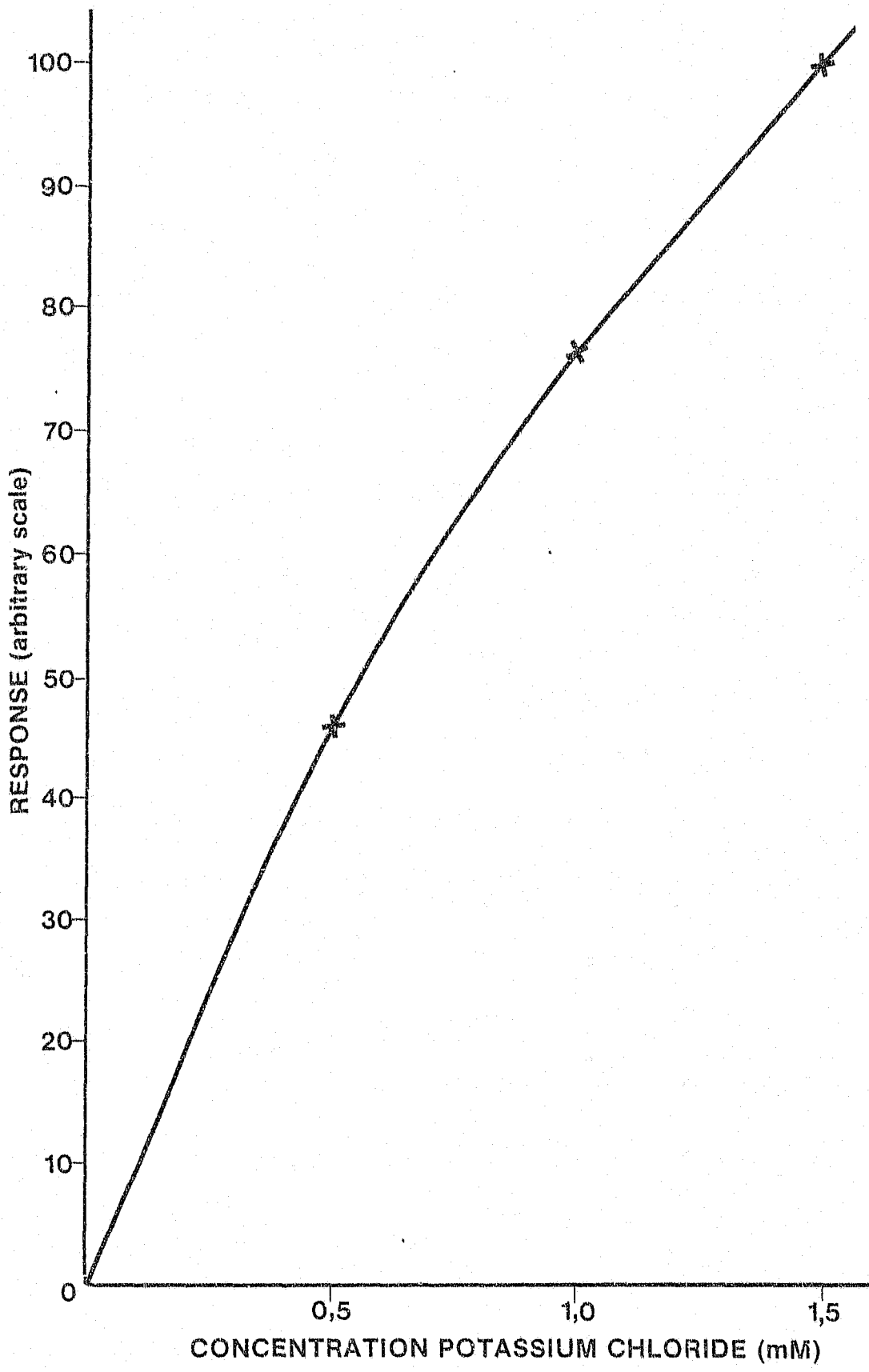
7.1 Calculations

The purpose of this part of the investigation was to study the changes in the levels of potassium in rat liver slices in normal and adrenalectomised rats with and without the addition of steroids (hydrocortisone and β-methasone) at zero time and over a 48 hour incubation.

Content is expressed as μmol of potassium present per gram of liver

FIG. 6

A calibration curve relating response of flame photometer against potassium chloride



III RESULTS

The work is divided into three parts :

(1) The effects of hormones on serine dehydratase activity *in vivo*

(2) The effects of hormones on serine dehydratase in liver slices in culture

(3) The morphological characteristics of the slices that bear on the activities of serine dehydratase observed.

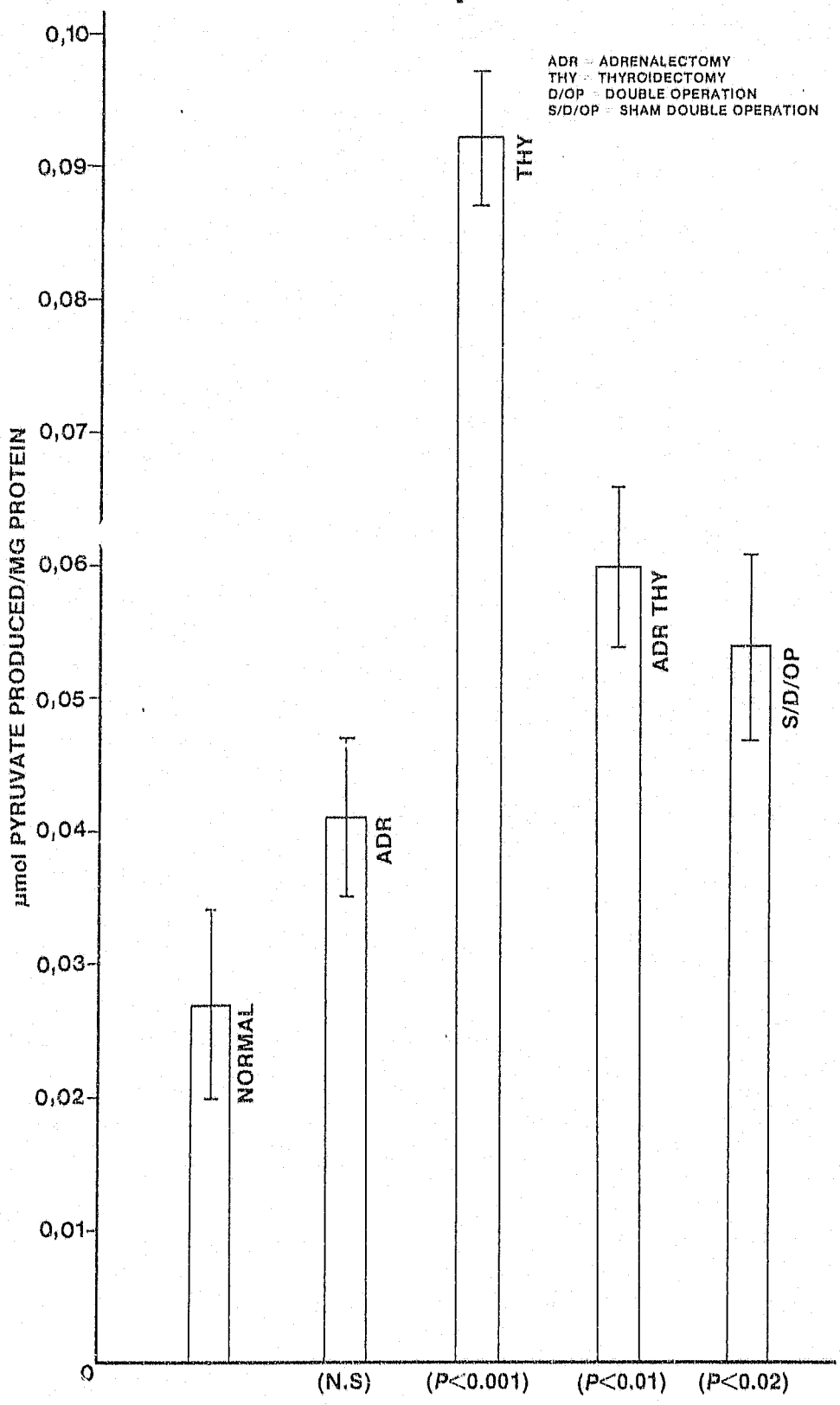
1.1 Effects of adrenalectomy, thyroidectomy, adrenalectomy and thyroidectomy (double operations) and sham operations on the activity of hepatic serine dehydratase

Groups of rats were adrenalectomised, thyroidectomised, doubly operated and double sham operated as already described. Thyroidectomy was performed at least 15 days prior to commencements, whereas adrenalectomy was done at least 6 days before killing and after thyroidectomy.

There was a marked increase in enzyme activity in thyroidectomised and double operated rats, although the activities in operated rats were much higher than the control non-operated normal animals (Fig.7). On statistical analysis the effect of adrenalectomy was not significant (N.S.) while thyroidectomy ($P < 0.001$), double operations ($P < 0.01$) and sham double operations ($P < 0.02$) were all significantly different

FIG. 7

Effect of adrenatectomy, thyroidectomy, double operations, sham double operations on the activity of serine dehydratase in vivo



Groups of rats were adrenalectomised, thyroidectomised, double operations and sham double operations. Thyroidectomy was performed at least 15 days prior to the commencement of the experiments while adrenalectomy 6 days and after thyroidectomy in the case of double operations. In sham double operations the same procedure was performed for adrenalectomy and thyroidectomy without removing the adrenals or thyroid glands. The operated animals were maintained on a laboratory diet while the drinking water was supplemented with 0,9% NaCl and 0,1% Ca gluconate. On comparing the statistical significance of operated rats to normal rats, adrenalectomy was not significant (N.S), while thyroidectomy ($P<0,001$) adrenalectomy and thyroidectomy ($P<0,01$) and sham double operations ($P<0,02$) were significant.

from normal unoperated rats.

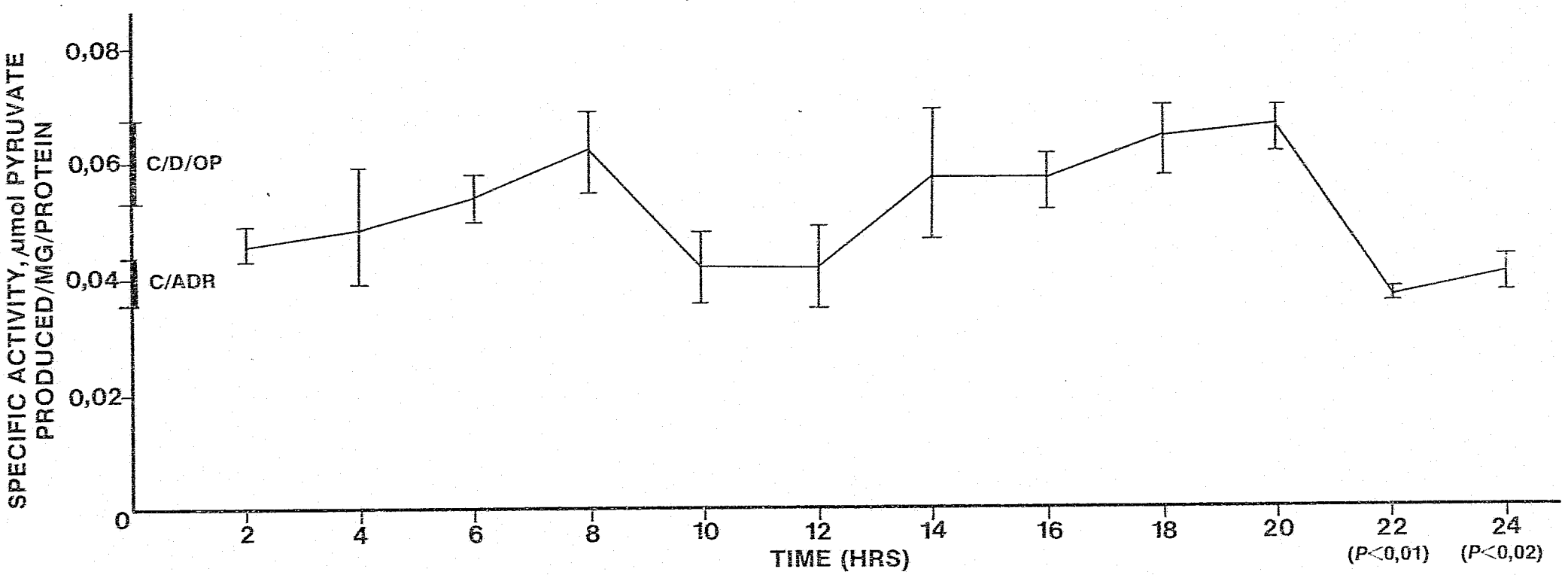
1.2 Effect of hydrocortisone (HC) on serine dehydratase activity in adrenalectomised and thyroidectomised rats

Before the effect of hormones on the enzyme levels *in vitro* could be tried it was necessary to test whether the hormone has an effect *in vivo* in the time period over which the *in vitro* experiments were to be carried out. Since the *in vitro* experiments were initially limited to 24 hours it was necessary to test whether effects on the enzyme could be produced over 24 hours *in vivo* in the experimental animals used for this study.

Rats previously adrenalectomised and thyroidectomised were injected intraperitoneally with hydrocortisone-21-sodium succinate (HC) administered as a Tween 20 suspension every 8 hours for 24 hours (6 mg/100 g body weight). The rats were killed every 2 hours after the commencement of the experiment up to 24 hours and the liver assayed for serine dehydratase activity (Fig. 8). On statistical analysis only the decreases at 22 ($P < 0.01$) and 24 hours ($P < 0.02$) were significant on comparing with control double operations. On comparing with control adrenalectomy only the inductions at 8 ($P < 0.05$) 18 ($P < 0.05$) and 20 hours ($P < 0.01$) were significant. The standard errors were high in some of the points investigated. According to Ishikawa and Nakajima (1970) hydrocortisone can induce activities in adrenalectomised rats but the effect of hydrocortisone seen here is in general not significant.

FIG. 8

Effect of hydrocortisone on the activity of serine dehydratase in adrenalectomised-thyroidectomised rats in vivo as a function of time



Rats previously adrenalectomised and thyroidectomised were injected intraperitoneally with HC (6mg/100g body weight). The HC injections were administered as a Tween 20 suspension every 8 hours for 24 hours. The rats were killed every two hours after the commencement of the experiment up to 24 hours and the livers removed and assayed for serine dehydratase activity.

The results are calculated as specific activity umol pyruvate formed/mg protein. On comparing the statistical significance with normal double operations at zero time, the effect of HC at 22 hours ($P<0,01$) and 24 hours ($P<0,02$) were significant. The rest were not significant (N.S).

C/D/OP = control double operations
C/ADR = control adrenalectomy

1.3 Effect of triiodothyronine (T_3) on the activities of the enzyme over a period of 24 hours in adrenalectomised and thyroidectomised rats

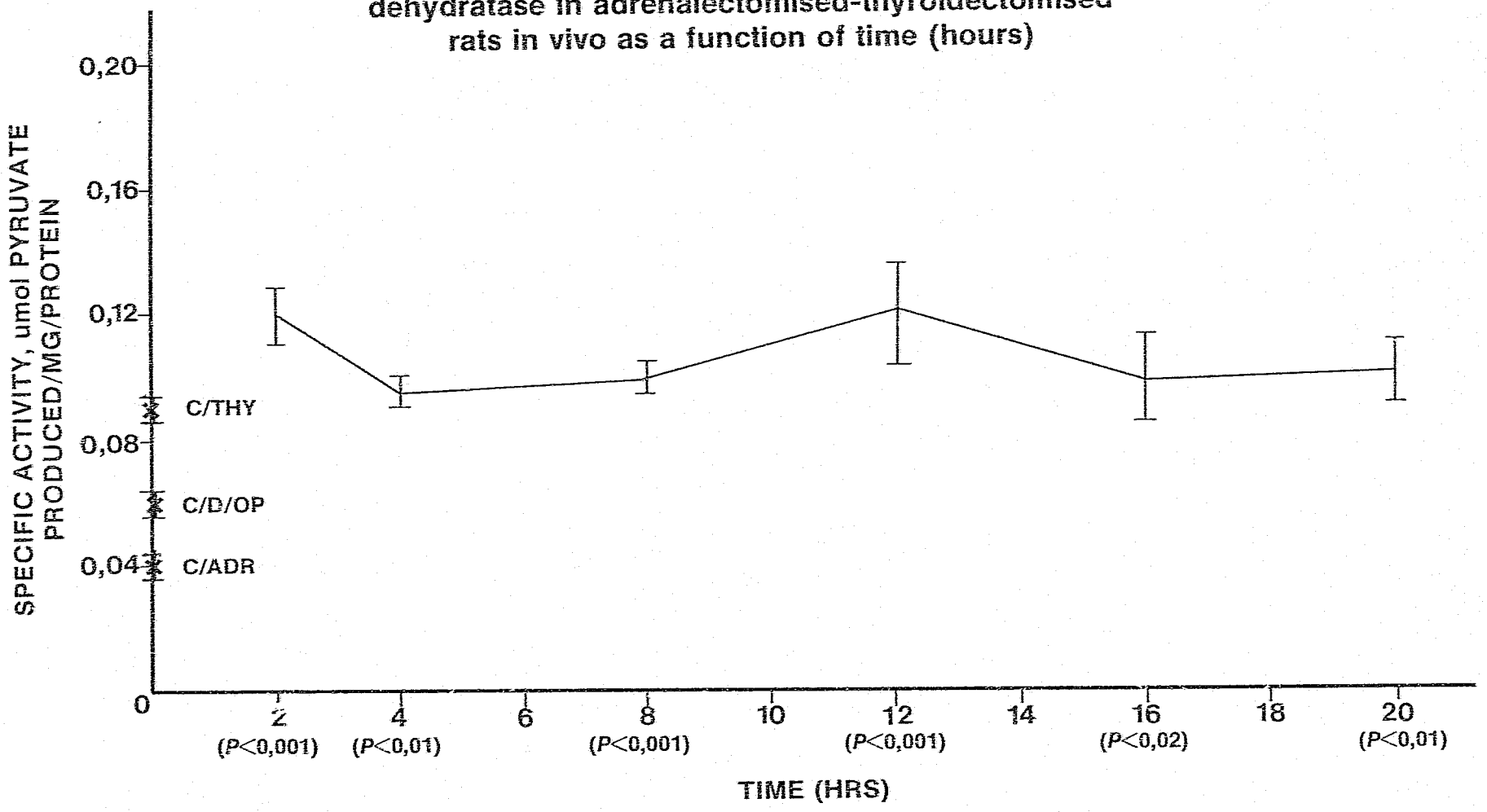
Rats previously adrenalectomised and thyroidectomised were injected with triiodothyronine (1 mg/100 g body weight) and serine dehydratase activity measured (Fig. 9). On statistical analysis the increase was found to be significant on comparison to control double operations suggesting an induction of enzyme. On comparison to control thyroidectomy the results were not significant except at 2 hours ($P < 0.05$). Ishikawa et al. (1965) found that administration of triiodothyronine for 5 days caused increased activities of the enzyme in adrenalectomised rats.

1.4. Effect of hydrocortisone and triiodothyronine on the activities of the enzyme over a period of 24 hours in adrenalectomised and thyroidectomised rats

Rats previously adrenalectomised and thyroidectomised were injected with hydrocortisone and triiodothyronine. The animals were killed at 2,4,8,12, 16 and 20 hours and activities measured (Fig. 10). There is a positive response of both hormones resulting in a marked increase in the activities of the enzyme at 4 and 16 hours ($P < 0.001$ and $P < 0.001$ respectively). The increase at 2,8,12 and 20 hours were also statistically significant but the extent of increase was less.

FIG. 9

Effect of triiodothyronine on the activity of serine dehydratase in adrenalectomised-thyroidectomised rats in vivo as a function of time (hours)

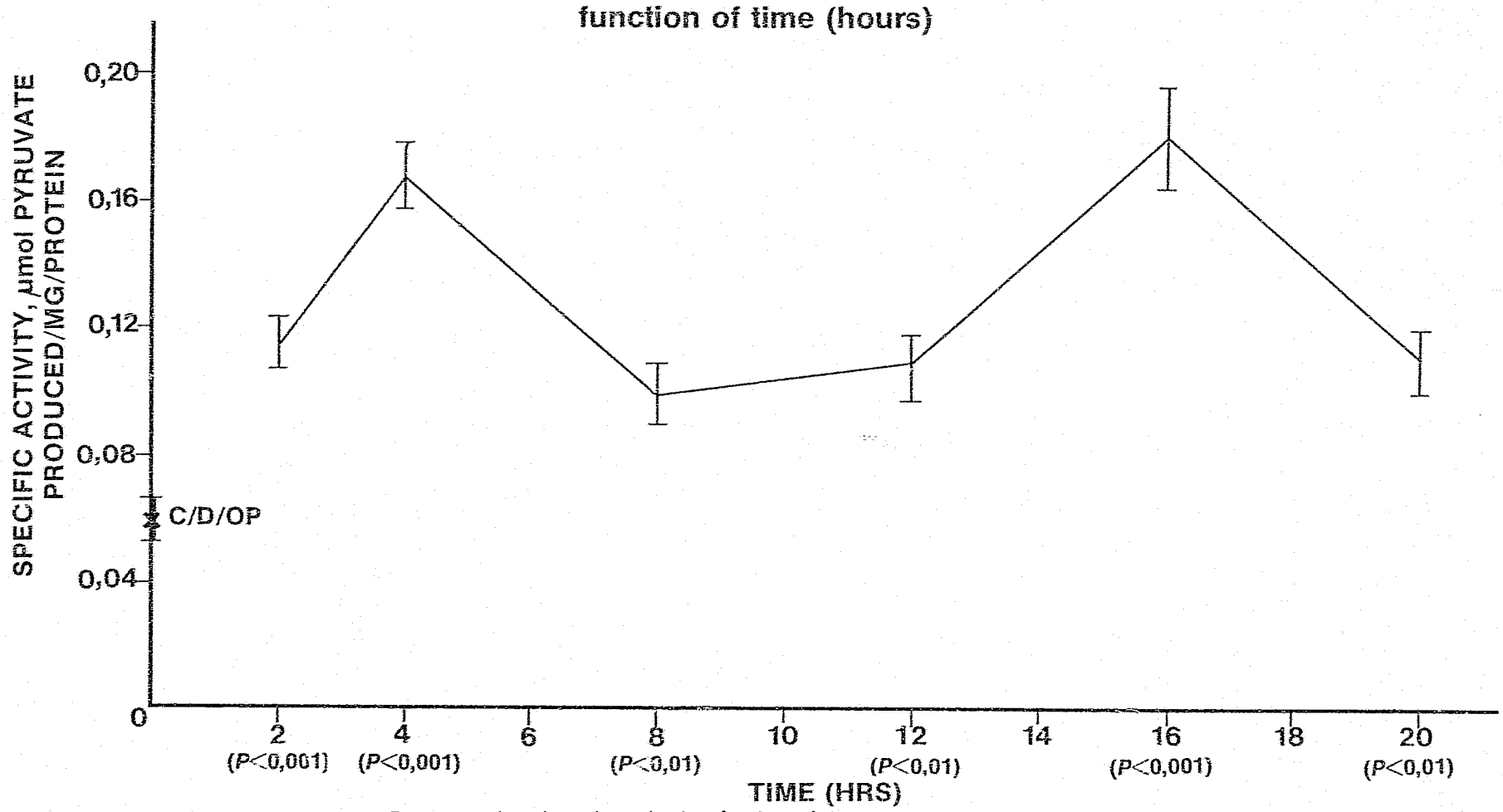


Rats previously adrenalectomised and thyroidectomised were injected intrapritoneally with Triiodothyronine (1mg/100g body weight). The rats were killed every four fours and the livers removed and assayed for serine dehydratase activity. The results shown are calculated as specific activity-umol pyruvate formed/mg protein.
Comparing the statistical significance of double operated animals at zero time to the effect of hormone on the enzyme activity as a function of time, they are all found to be significant.

C/D/OP = control double operations
C/ADR = control adrenalectomy
C/THY = control thyroidectomy

FIG. 10

Effect of hydrocortisone and triiodothyronine on the acitivity of serine dehydratase in adrenalectomised-thyroidectomised rats in vivo as a function of time (hours)



Rats previously adrenalectomised and thyroidectomised were injected intraperitoneally with HC (6 mg/100 gm body wt) and triiodothyronine (1mg/100g body weight). The rats were killed every four hours and the livers assayed for serine dehydratase activity. The results shown are as specific activity-umol pyruvate formed/mg protein.

Comparing the statistical significance of double operated rats at zero time to the effect of both hormones on the enzyme activity as a function of time, they are all found to be significant.

C/D/OP = control double operations

1.5 Activity of serine dehydratase and lactic dehydrogenase in normal and adrenalectomised rats maintained on fed and 24 hours fasted dietary regimes

Intact and adrenalectomised rats were put into two groups. One group was fed while the other was fasted for a minimum of 24 hours before measurement of serine dehydratase activity (Table 2). The results indicate that there was a marked increase in SDH activity in the fasted rat compared to the normal fed rat. Similarly in the adrenalectomised rat the same pattern occurs with an increase in the fasted rat compared to that of the fed ones. The results agree with published literature (Ishikawa and Nakajima, 1970; Suda, 1967). In these experiments lactic dehydrogenase was also assayed. There was a slight increase in the normal rats with fasting and also the same for adrenalectomised rats.

1.6 Activity of serine dehydratase and lactic dehydrogenase in adrenalectomised rats injected with hydrocortisone and maintained on food and fasted dietary regime

Rats were put into two groups, one was fed while the other was fasted for 24 hours before the animals were killed. Both groups were injected intraperitoneally with hydrocortisone (0.75 mg HC in 0.5 ml saline). Injections (0.5 ml) were given at intervals of 6 hours. The rats were killed 6 hours after the last injection and enzyme activities measured

TABLE 2

Effects of food, 24 hour fasting and hydrocortisone treatment on the activities of serine dehydratase (SDH) and lactic dehydrogenase (LDH) in normal and adrenalectomised rat liver

	SDH	LDH
ACTIVITY (UNITS) μmol NAD produced/min/gm liver		
Control - Normal + Food	0.62* ± 0.01	312 * ± 10
Control - Normal + 24 hour fasting	2.74 ± 0.17	361 ± 14
Adrenalectomised and fed	0.76** ± 0.04	323 ± 5
Adrenalectomised and fasted 24 hours	2.76 ± 0.20	345 ± 21
Adrenalectomised fed plus HC	1.30*** ± 0.07	386 ** ± 9
Adrenalectomised and fasted plus HC	2.20 ± 0.22	287 ± 7

For details see next page

Rats were adrenalectomised 6 days prior to commencement of experiments. Where indicated rats were injected intraperitoneally with hydrocortisone 0.75 mg HC in 0.5 ml normal saline (0.9%) per animal, at 6 hourly intervals for 1 day and the animal was killed 6 hours after the last injection (0.5 ml/injection).

Each value is the mean ± S.E.M. of nine observations. P values were calculated in order to find any statistical significance. For serine dehydratase * indicates $P < 0.001$ with respect to normal fasted, ** $P < 0.01$ with respect to normal fed. On comparing adrenalectomised fed to adrenalectomised fasted $P < 0.001$. *** $P < 0.001$ with respect to the same in fasted animals. Adrenalectomised fed to adrenalectomised fed plus HC $P < 0.001$, while the same for the fasted animals was not significant.

For lactic dehydrogenase * $P < 0.02$ with respect to normal fasting. Adrenalectomised fed an fasted with respect to normal fed and fasted were not significant. Adrenalectomised fed to adrenalectomised fasted was not significant while ** $P < 0.001$ with respect to the same in fasted animals. Adrenalectomised fed compared to adrenalectomised fed plus HC $P < 0.001$ while adrenalectomised fasted to adrenalectomised fasted plus HC $P < 0.02$.

(Table 2). The results indicate a marked increase in the activity for SDH in the fed animals. Ishikawa and Nakajima (1970) found that on injection of hydrocortisone to adrenalectomised fed rats there was a 10% increase in activity while in fasted rats the increase was nearly two-fold. In this experiment a decrease in enzyme activity on injection of hydrocortisone to fasted adrenalectomised rats is seen. For LDH an increase in activity in the fed rats was found while in the fasted rats hydrocortisone decreased activity. Wick (1968) found that hydrocortisone administration did not alter LDH activity in foetal rat liver.

2.0 In vitro effect of hormones and dietary regimes on serine dehydratase and lactic dehydrogenase activity of slices of adult rat liver over a 24 hour and 48 hour incubation

Rat liver slices were prepared and cultured according to the method as mentioned in Materials and Methods. The slices were infused with 95% oxygen and 5% carbon dioxide. The atmosphere was renewed after 12 or 24 hours of incubation at the same time as hormonal additions were made to the medium mixture. The slices were maintained in an incubator at 37°C.

2.1 Effect on the activities of serine dehydratase and lactic dehydrogenase over a 24 hour incubation period

Normal rat liver slices were cultured for 24 hours at 37°C. Groups of

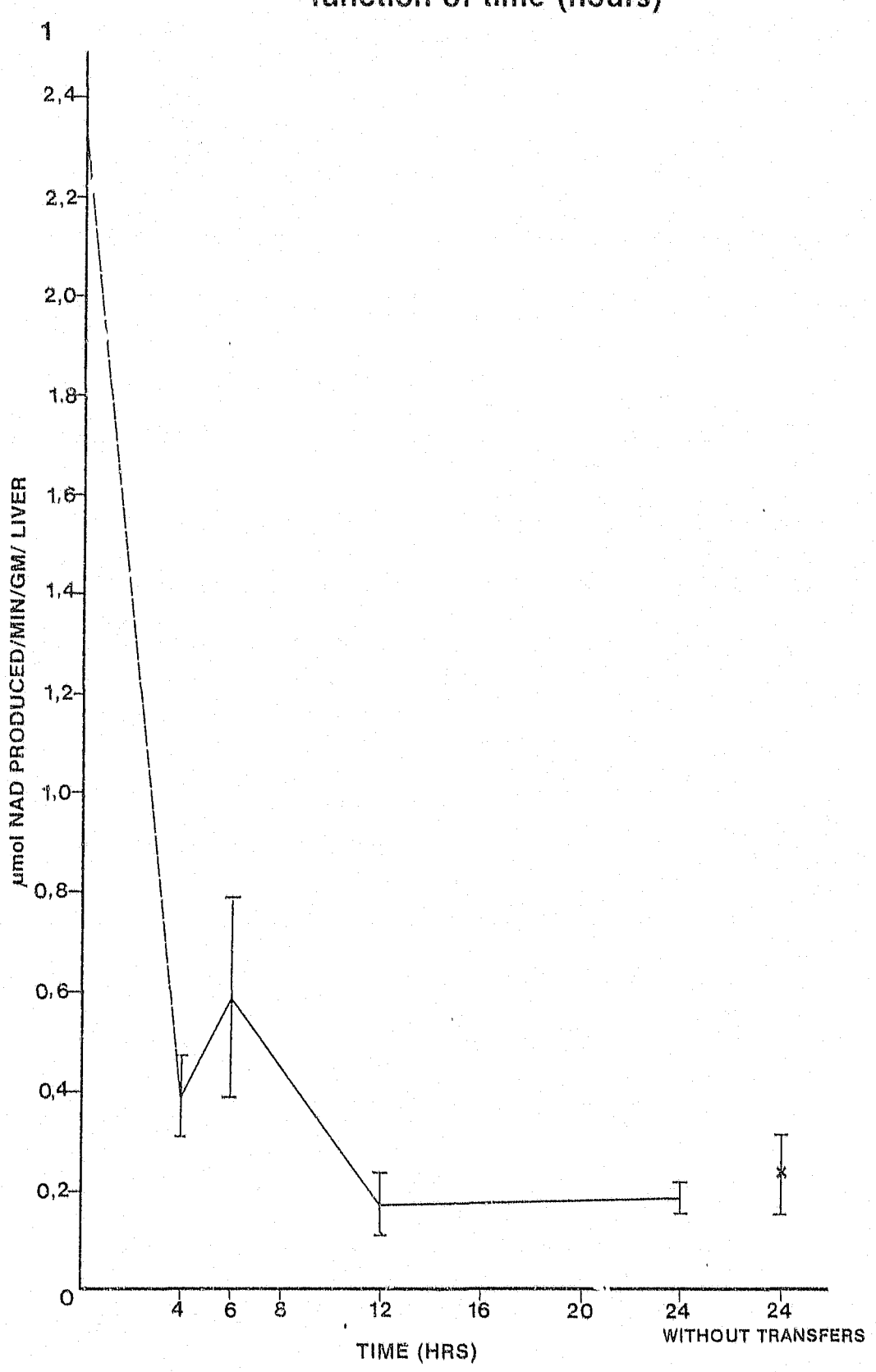
cultured slices were removed at different times and assayed for SDH and LDH. Activities were recorded after 0, 4, 6, 12 and 24 hour incubation (Fig. 11). A culture without a 12 hour transfer, i.e. a straight 24 hour incubation, was also assayed. The results indicate a large drop in SDH enzyme activity from zero time (*in vivo*) to 4 hours of culture. From 4 hours the levels fall further to 12 hours after which it is relatively constant. A straight 24 hour culture without transfers showed levels that were not any different from a two 12 hour culture. No SDH activity was found in the medium.

In order to see if the presence of substrate in the medium would stabilise the enzyme, preventing such a big decrease in enzyme levels from *in vivo* to *in vitro* condition, 10 mM of serine was added to the medium. Figure 12 shows that the decrease was still the same.

Lactic dehydrogenase activity also decreased significantly from zero time during incubation for 4 hours ($P < 0.001$). From 4 hours there was a gradual decrease to 6, 12 and 24 hours (Fig. 13). A straight 24 hours culture without 12 hour transfers was no different than the two 12 hour cultures. On assaying the medium of a 4 hour incubation sample considerable activity was present. Activity steadily increased from 4 to 12 hours and then decreased to 24 hours (Fig. 14). A straight 24 hour culture medium not surprisingly showed a higher level of enzyme activity compared to two 12 hour ones. MacDonnel *et al*. (1975) found

Fig 11.

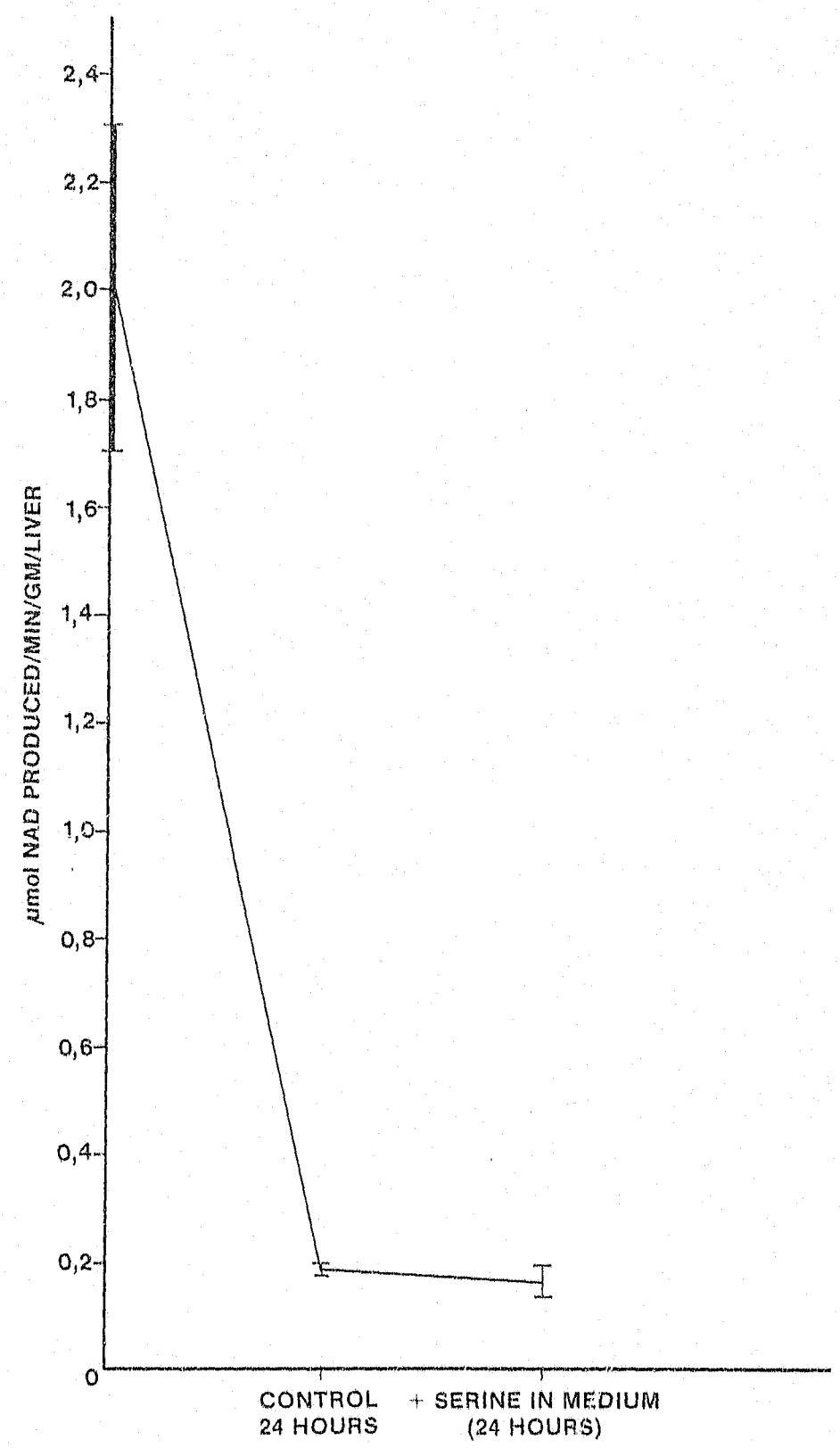
Serine dehydratase activity in liver slices as a function of time (hours)



Rats were killed and the livers removed The slices were cultured in normal medium and incubated over a 24 hour period at 37° C. Samples were removed at different incubation times and the slices and medium assayed for enzyme activity.

FIG. 12

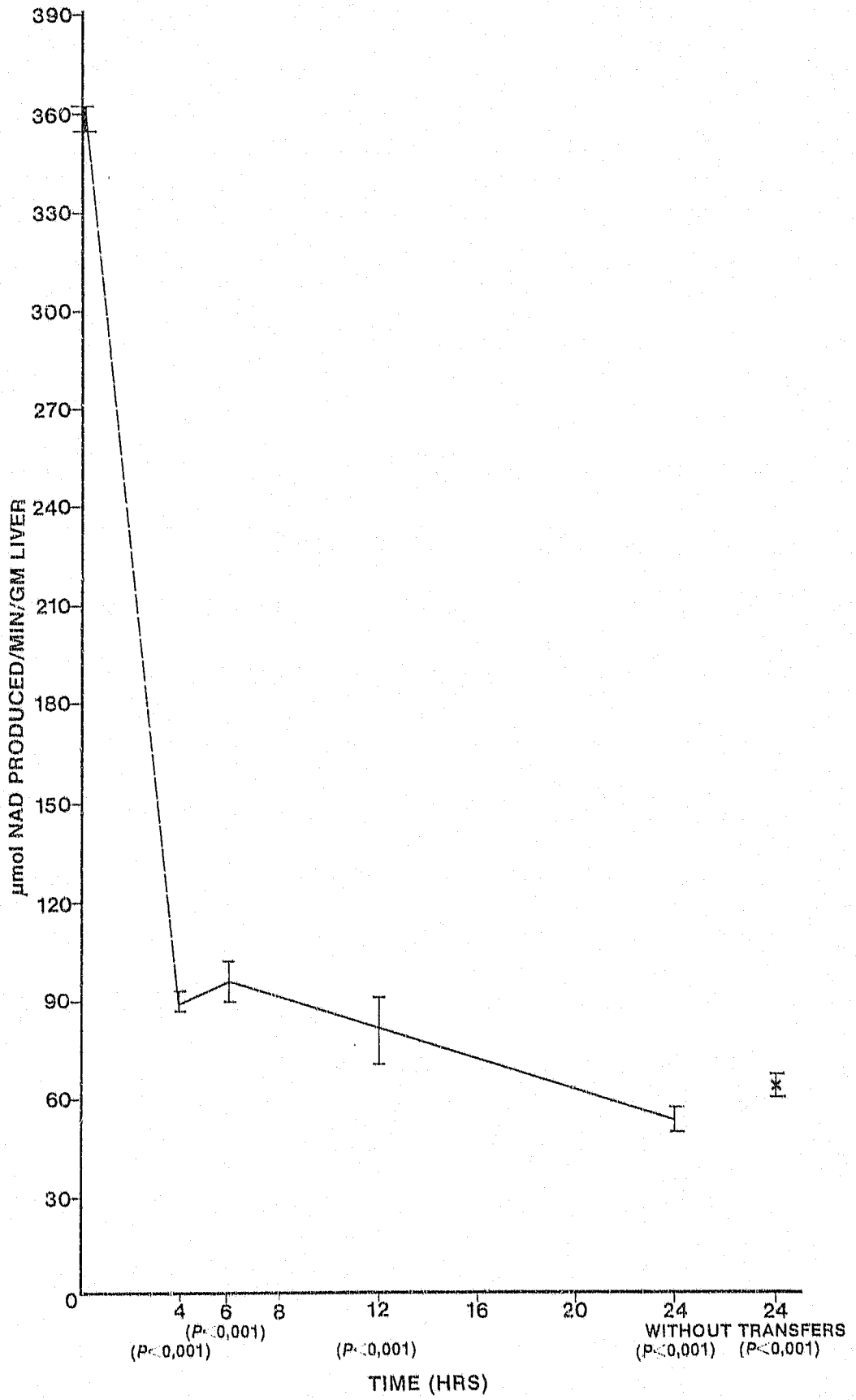
Effect of serine in medium mixture (10 mM) on the activity of serine dehydratase in adult rat liver slices



Rats were killed and the liver slices cultured in normal medium and medium containing (10 mM) serine. Slices cultured and incubated for 24 hours at 37°C with a 12 hour transfer to fresh medium and medium containing serine. The atmosphere was also renewed. A zero time level was recorded using fresh liver and assayed for enzyme activity.

FIG. 13

Lactic dehydrogenase activity in liver slices as a function of time (hours)

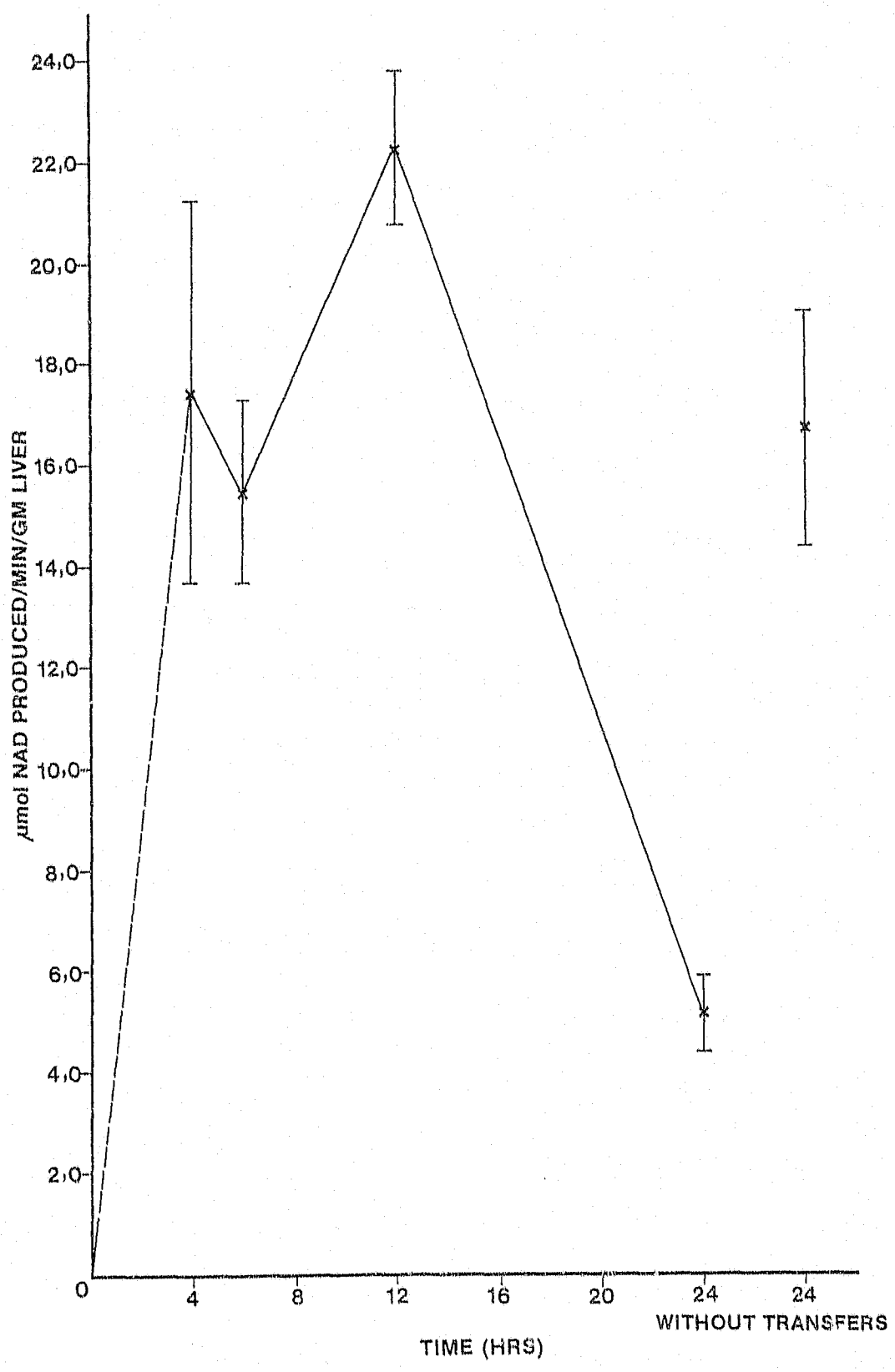


The slices were cultured in normal medium and incubated over a period of 24 hours at 37° C. Samples were removed at different incubation times and the slices and medium assayed for enzyme activity.

Comparing statistical significance of a zero time sample (in vivo) to samples incubated over a period of 24 hours at 37° C, they all are significant as shown by the *P* values on the graph. The statistical analysis of LDH (tissue) and LDH (medium) results were found to be statistically significant (T 6,875).

FIG. 14

Lactic dehydrogenase activity in medium as a function of time (hours)



that 20 day old foetuses, cultured for 48 hours in the absence of serum, released 70% of their total soluble protein content in the medium. In the presence of serum their loss was 60%. They also found that LDH was released into the medium.

2.2 Effect of hydrocortisone on the activity of serine dehydratase in slices from normal and adrenalectomised rats

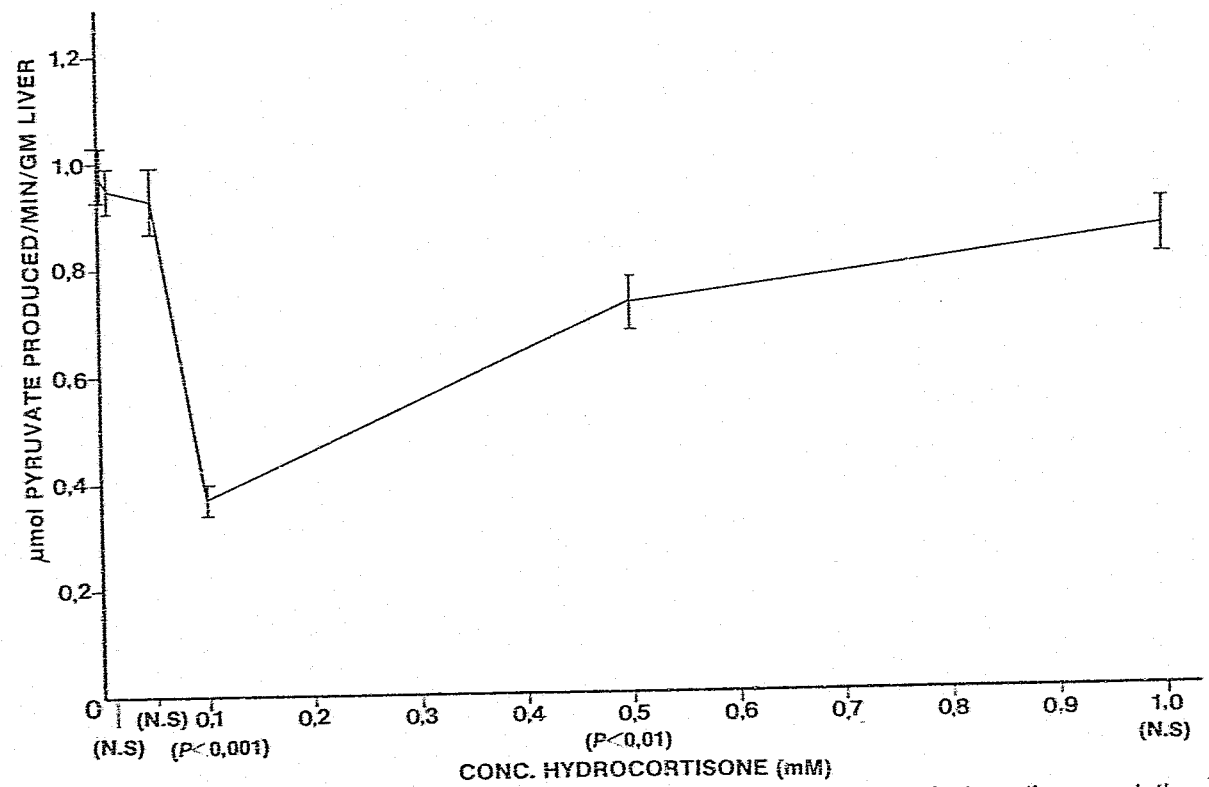
Groups of cultures were prepared, each having two sets of cultures in control medium (without hormone) and duplicate sets of cultures with medium containing the hormone in concentrations from 0.01 mM to 1 mM. The slices were put in normal medium for the first 12 hours and then transferred to media containing hormone for a further 12 hours of incubation at 37°C. The results (Fig. 15) appear to show that 0.1 mM and 0.5 mM HC depresses SDH activity while 1.0 mM restores it towards the levels in the absence of HC in normal rats. Figure 16 shows the effect in adrenalectomised rats where it appears that at a concentration of 0.5 and 1.0 mM HC depresses the activity of the enzyme. Just as in figure 15 no induction of the enzyme is seen here.

2.3 Effect of β-methasone or the activity of serine dehydratase in slices from normal and adrenalectomised rat liver

β-Methasone is a synthetic glucocorticoid, 40 times more active than hydrocortisone.

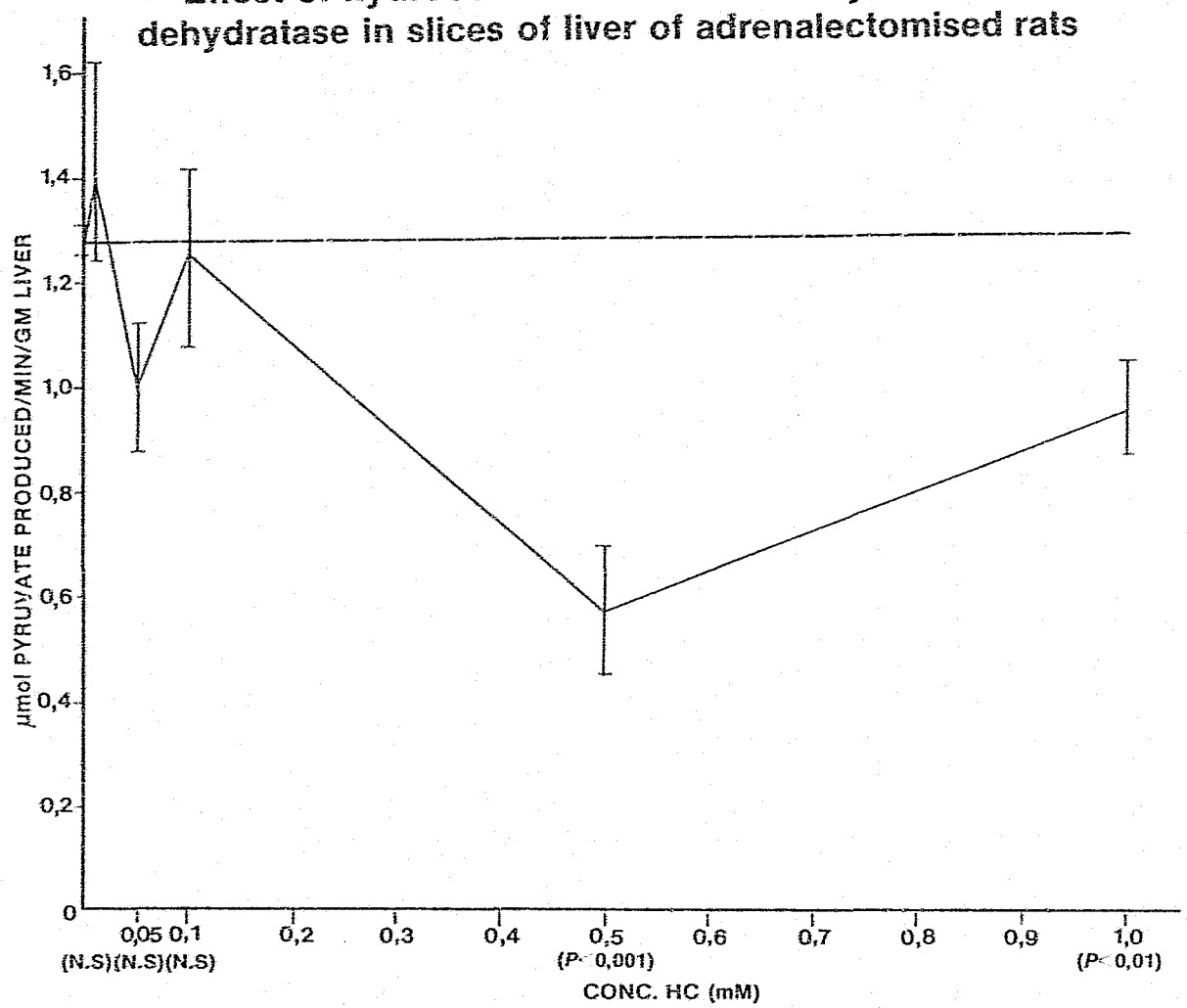
FIG. 15

Effect of hydrocortisone on the activity of serine dehydratase in normal rat liver slices



Liver slices were cultured at 37° C for 24 hours. For the first 12 hours the cultures were maintained in normal medium.
For the next 12 hours the cultures were transferred to medium containing hydrocortisone and the atmosphere was also renewed at this stage.
The statistical significance (*P* values) have been shown on the graph.

FIG. 16



Rats were adrenalectomised 6 days prior to the commencement of experiments and fed normal laboratory food, the drinking water was supplemented with 0.9% saline. They were killed and their livers quickly removed. The liver slices were cultured in normal medium for the first 12 hours and then transferred to medium containing hydrocortisone for the next 12 hours. The atmosphere was renewed after the first 12 hours and incubated at 37°C.
The statistical significance (*P* values) have been shown on the graph.

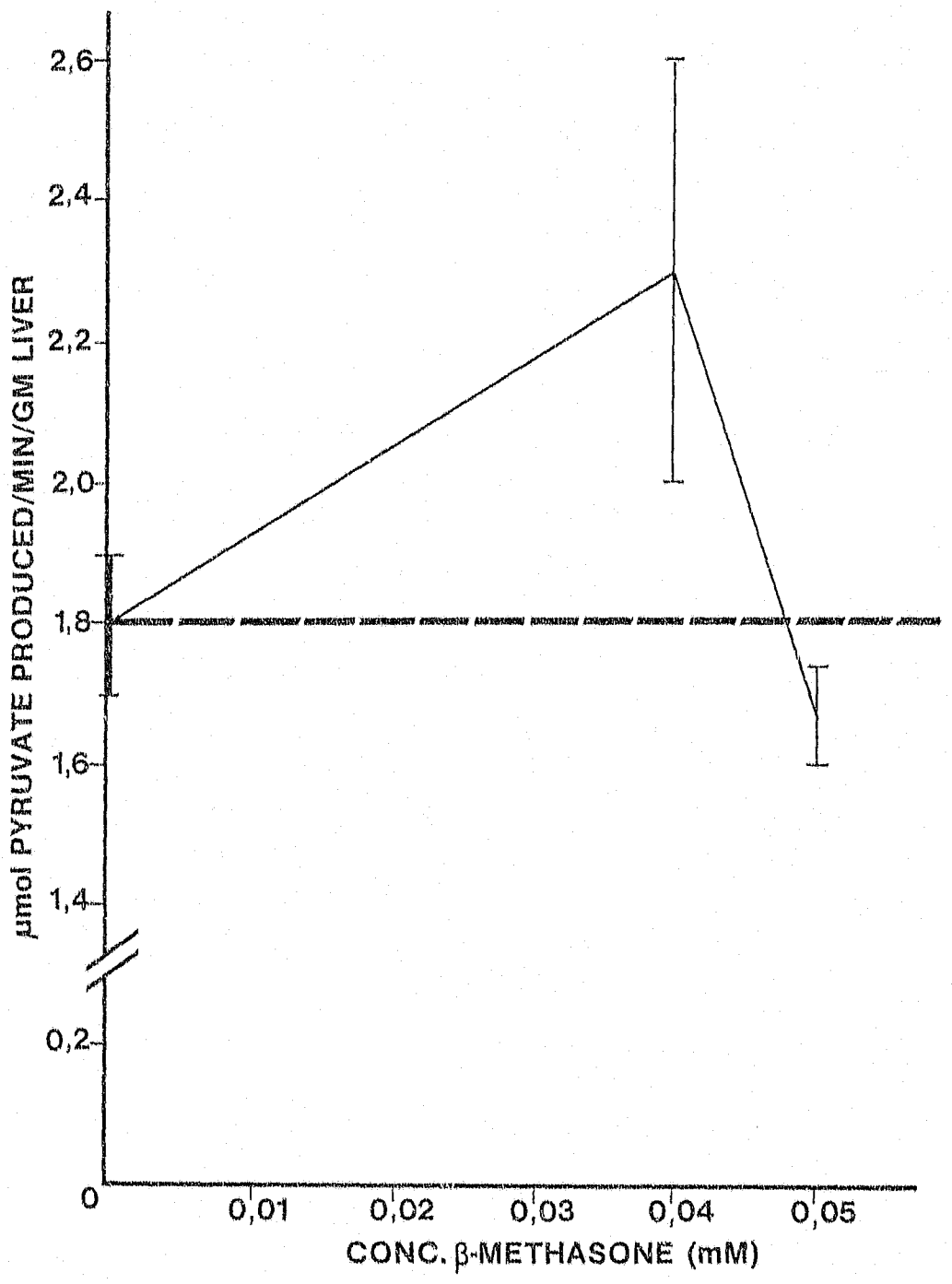
Groups of cultures were prepared, each having two sets in control medium and duplicate sets of cultures with medium containing hormone in concentrations from 0.01 mM to 0.1 mM. The slices were cultured for the first 12 hours in normal medium (without hormones) and then transferred to media containing hormone for the next 12 hours of incubation at 37°C. In the normal rats there was no significant effect of β-methasone (Fig. 17). In the adrenalectomised rats there was a significant decrease in enzyme activety at a concentration of 0.06 mM β-methasone ($P < 0.05$) while the remaining concentrations are not significant (Fig. 18).

2.4 Activity of serine dehydratase and lactic dehydrogenase in normal and adrenalectomised rats maintained on a food and fasted dietary regime

Rat liver slices from normal and adrenalectomised rats, previously fed or starved for 24 hours before commencement of experiments, were cultured over a period of 24 hours. The cultures were transferred after 12 hours to fresh medium and cultured for another 12 hours at 37°C. The slices were homogenised and assayed for enzyme activity. As shown earlier in Figure 11 and Table 2 there was a very large decrease in enzyme activities from *in vivo* to *in vitro*. As shown in Table 3 there was no significant difference in activity in the normal rats between fed and fasted dietary regimes, though in the adrenalectomised rats there was a significant difference ($P < 0.01$).

FIG. 17

Effect of β-methasone on the activity of serine dehydratase in normal rat liver slices

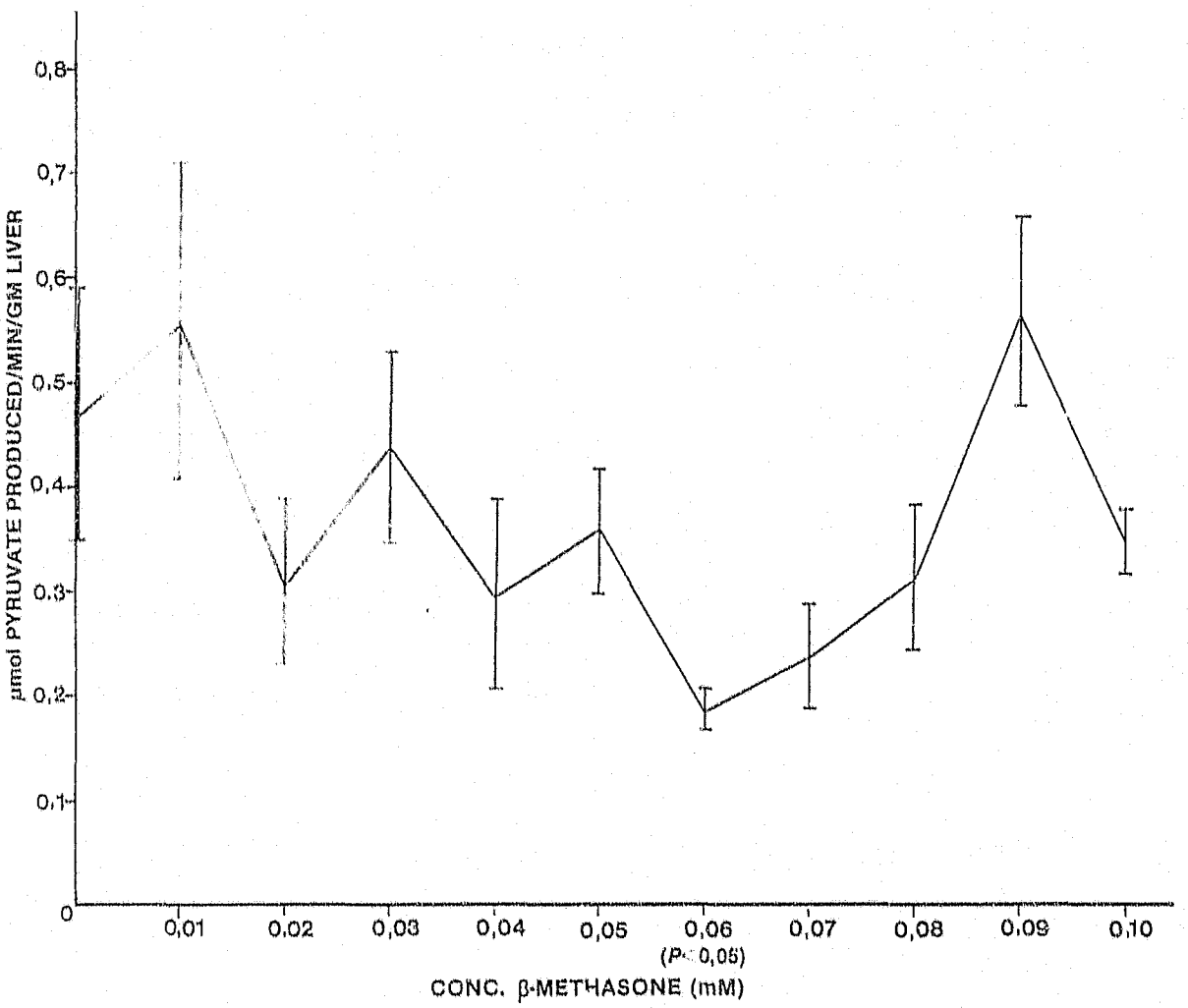


Liver slices were cultured for 24 hours at 37°C. Initially the cultures were maintained for 12 hours in normal medium after which they were transferred to medium containing β-methasone (0,04 and 0,05 mM respectively) for 12 hours. The atmosphere was renewed after the initial 12 hours incubation.

On comparing to the zero time (in vivo) level there seems to be an induction at 0,04 mM but on statistical analysis (P values) of both concentrations they were found to be not significant (N.S).

FIG. 18

Effect of β-methasone on the activity of serine dehydratase in adrenalectomised rat liver slices



Liver slices were cultured for 24 hours at 37°C. For the first 12 hours the cultures were maintained in normal medium. For the next 12 hours the cultures were transferred to medium containing hormone (β-methasone, dose-curve 0,01 mM — 0,10 mM). At this stage the atmosphere was renewed. The statistical significance (*P* values) are shown on the graph.

For LDH there was a decrease in activities in normal rats between fed and fasting. In the adrenalectomised rats there was an increase in activities in the fed rats to that of the fasted ones. From *in vivo* to *in vitro* there was a large decrease in activity (Table 3).

2.5 Effect of hydrocortisone on the activity of serine dehydratase and lactic dehydrogenase in slices from fed and fasted adrenalectomised rats

Activity of SDH was slightly higher in the fasted animal compared to the fed oner (Table 3). As in earlier experiments there was a remarkable drop in SDH activity from *in vivo* to *in vitro* systems. For LDH the animals kept on a fasted dietary regime under the influence of HC were found to have lower activities than the fed ones. Wicks (1968) found that in foetal rat liver organ culture, the enzyme activity of LDH was not altered after a 17 hour exposure to HC.

2.6 Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in slices of adrenalectomised rats as a function of incubation time

Slices were cultured in medium containing β-methasone (0.01 mM final concentration) and incubated for 48 hours at 37°C. Control samples were cultured in normal medium. Samples were removed at 6, 12, 24 and 48 hours and the slices assayed for serine dehydratase activity.

On comparing with the control values at each incubation time there is no induction of the enzyme by β-methasone (Table 4). The experimental values are all below the normal controls.

On assaying a sample of the medium for each incubation time the presence of enzyme in the medium was found indicating that some of the enzyme had leaked out of the cell. It was fairly constant up to 24 hours.

2.6.1 Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in adrenalectomised rat liver slices as a function of incubation time

After incubating the samples for 48 hours in normal medium as in 2.6 the slices were transferred to medium containing the hormone (β-methasone, final concentration 0.01 mM) and incubated at 37°C. Samples were removed at 2, 4, 6, 8 and 10 hours (after initial 48 hour incubation) and slices assayed for serine dehydratase activity.

On comparison with a 48 hour control value the enzyme was not found to have been induced by the hormone over a further 10 hour incubation. No significant activity was found on assaying a portion of the medium.

2.6.2 Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in normal rats as a funtion of incubation time

Slices were cultured in normal medium and incubated for 48 hours at 37°C. After 48 hours the slices were transferred to medium containing

TABLE 3

Effects of food 24 hours fasting and hydrocortisone treatment on the activities of serine dehydratase (SDH) and lactic dehydrogenase (LDH) in liver slices from normal and adrenalectomised rats

Slices made from liver of	SDH	LDH
ACTIVITY (UNITS) µmol NAD produced/min/gm liver		
Normal fed	0.106 ± 0.008	89.6 ± 7.4
Normal fasted	0.114 ± 0.016	28.7 ± 1.8
Adrenalectomised and fed	0.134 ± 0.014	147.0 ± 12.4
Adrenalectomised and fasted 24 hours	0.079 ± 0.003	41.0 ± 3.8
Adrenalectomised, fed plus HC *in vitro*	0.049 ± 0.004	56.4 ± 0.6
Adrenalectomised fasted plus HC *in vitro*	0.084 ± 0.013	18.1 ± 1.1

For details see next page

Rats were adrenalectomised 6 days prior to commencement of experiments and were maintained on either a normal laboratory or 24 hour (prior to start of experiment) fasting dietary regime. The liver slices were cultured in normal medium for the first 12 hours then transferred to medium containing hormone, the atmosphere was renewed and the cultures incubated for a further 12 hours at 37°C. At the end of the incubation time the slices were removed and assayed for enzyme activity.

Each value is the mean ± S.E.M. of nine observations. P values were calculated in order to find any statistical significance. For serine dehydratase, normal fed with repect to normal fasted, normal fed to adrenalectomised fed and normal fasted to adrenalectomised fasted were all not significant. Adrenalectomised fed to adrenalectomised fasted $P < 0.01$, adrenalectomised fed plus HC to the same in fasted animals $P < 0.02$. Adrenalectomised fed to adrenalectomised fed plus HC $P < 0.02$ while adrenaelectomised fasted to adrenalectomised fasted plus HC was not significant.

For lactic dehydrogenase normal fed to normal fasted $P < 0.001$, normal fed to adrenalectomised fed $P < 0.01$ and normal fasted to adrenalectomised fasted $P < 0.02$. Adrenalectomised fed to adrenalectomised fasted $P < 0.001$ adrenalectomised fed plus HC to the same in fasted animals $P < 0.001$. Adrenalectomised fed to adrenalectomised fed plus HC $P < 0.001$ while adrenalectomised fasted to adrenalectomised fasted plus HC $P < 0.001$.

TABLE 4

Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in slices from fed adrenalectomised rats

SDH ACTIVITY: µmol/NAD produced/min/gm liver					
Incubation time (hours)	0	6	12	24	48
Slices Control	1.98 ± 0.028	0.09 ± 0.002	0.12 ± 0.003	0.07 ± 0.004	0.07 ± 0.002
Medium		0.09 ± 0.003	0.07 ± 0.003	0.045 ± 0.002	0.07 ± 0.003
Slices 0.01 mM β- methasone		0.08 ± 0.003	0.08 ± 0.003	0.06 ± 0.003	0.04 ± 0.001
Medium		0.12 ± 0.011	0.09 ± 0.003	0.08 ± 0.001	0.15 ± 0.011

For details see next page

Adult rats adrenalectomised 6 days previously were killed and livers removed. The slices were cultured in normal medium (control) or medium containing hormone (0.01 mM final concentration) and incubated for 48 hours at 37°C. Cultures were removed at 6, 12, 24 and 48 hours and assayed for enzyme activity. The medium for both the experimental and control cultures was also examined for enzyme activity. The atmosphere was renewed at 24 and 48 hours incubation.

Each value is the mean $\pm$ S.E.M. of three observations. P values were calculated in order to find any statistical significance. In the control sample on incubation for 6, 12, 24 and 48 hours $P < 0.001$ respectively with respect to zero time control. The addition of hormone to the medium and incubating for 6 hours (N.S.), 12 hours significant decrease $P < 0.01$, 24 hours (N.S.) and 48 hours a significant decrease $P < 0.01$.

0.01 mM hormone and incubated for a further 8 hours at 37°C. The atmosphere was renewed after 24 and 48 hours. Samples were removed after 2, 6 and 8 hours of incubation and the slices and medium assayed for enzyme activity.

On comparing the results obtained (Table 5) there was a drop in activity from an initial 48 hour control incubation to 2 hours incubation (hormone). There is an induction at 6 hours by the hormone which then decreased at 8 hours incubation.

Enzyme activity steadily increased in the medium from an initial 48 hour control sample to an 8 hour incubation with hormone.

2.7 Effect of hydrocortisone (HC) on the activity of serine dehydratase in liver slices from adrenalectomised rats as a function of incubation time

Slices were cultured in medium containing the hormone and incubated at 37°C. Samples were removed at 6, 24 and 48 hours and the slices assayed for serine dehydratase activity. For each experimental sample there was also a control sample cultured in normal medium (Table 6). On comparison with control values the hormone did not induce the enzyme activity. The medium did show the presence of activity at 6 and 48 hours incubation.

2.8 Effect of dbcAMP (1 mM, 2 mM) on the activity of serine dehydratase over a 48 hour incubation in rat liver slices

TABLE 5

Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in normal rat liver slices after an initial 48 hour incubation as a function of incubation time

SDH ACTIVITY: µmol NAD produced/min/gm liver				
Incubation time with β-methasone	0	2	6	8
Slices	0.27 ± 0.005	0.21 ± 0.03	0.39 ± 0.036	0.36 ± 0.002
Medium	0.04 ± 0.003	0.09 ± 0.002	0.12 ± 0.002	0.12 ± 0.005

Normal adult rats maintained on a laboratory diet were killed and the livers removed. The slices were cultured in normal medium and incubated for 48 hours at 37°C. A tissue and medium sample of 48 hour (0) incubation was assayed for enzyme activity. The slices were transferred to medium containing hormone (0.01 mM final concentration) and infused with fresh atmospheric mixture (95% oxygen and 5% carbon dioxide) and incubated for a further 8 hours. Samples were removed at 2, 6 and 8 hours and the slices and medium assayed for activity.

Each value is the mean ± S.E.M. of three observations. P values were calculated in order to find any statistical significance. After the initial incubation for 48 hours the cultures were incubated for a

further 8 hours in medium containing hormone. At 2 hours there was a significant decrease in enzyme activity $P < 0.001$ while at 6 and 8 hours there was an induction of enzyme activity $P < 0.001$ respectively.

TABLE 6

Effect of hydrocortisone on the activity of serine dehydratase in slices from adrenalectomised rats

SDH ACTIVITY: µmol NAD produced/min/gm liver				
Incubation time (hrs)	0	6	24	48
Control Slices	1.97 ± 0.03	0.09 ± 0.002	0.07 ± 0.003	0.07 ± 0.003
Medium		0.09 ± 0.002	0.04 ± 0.003	0.07 ± 0.003
2×10^{-6}M hydrocortisone Slices		0.06 ± 0.003	0.06 ± 0.001	0.06 ± 0.002
Medium		0.12 ± 0.002		0.18 ± 0.003

For details see next page

Adult rats (adrenalectomised 6 days previously) were killed and livers removed. The slices were cultured in normal medium (control) or medium containing hormone (2×10^{-6}M final concentration of HC) and incubated at 37°C for 48 hours. Control and experimental cultures were removed at 6, 24 and 48 hours and the slices assayed for enzyme activity. The medium for both the control and experimental cultures was also examined for enzyme activity. The atmosphere was renewed at 24 and 48 hours.

Each value is the mean $\pm$ S.E.M. of three observations. P values were calculated in order to find any statistical significance. On incubation for 6, 12, 24 and 48 hours there was a significant decrease in enzyme activity with respect to zero time control $P < 0.001$ respectively. On the addition of hormone to the medium and incubating for 48 hours, there was no induction of the enzyme, rather a significant decrease, at 6 hours $P < 0.01$, 24 hours not significant and 48 hours $P < 0.05$.

Slices were cultured for 24 hours in normal medium. Some slices were removed for 24 hour assay while the rest were transferred to normal medium and media containing 1 mM and 2 mM (final concentration) of dbcAMP. The enzyme was induced with both concentrations of dbcAMP (Table 7).

2.9 Effect of dbcAMP (2 mM) on the activity of serine dehydratase in rat liver slices as a function of incubation time

Rats maintained on laboratory food were killed and the livers quickly removed. The slices were cultured in medium containing hormone (2 mM final concentration) and incubated at 37°C. Control cultures in normal medium were also incubated along with the experimental ones. Samples were removed at 6, 12, 24 and 48 hours and the slices assayed for serine dehydratase activity (Table 8). On comparing the results obtained with control values the enzyme appears to be induced by the hormone at 12 and 24 hours. The medium of both the control and the experimental samples contained marked levels of the enzyme.

2.10 Effect of glucagon (250 µg/ml) and dbcAMP (2 mM) on the activity of serine dehydratase in adult rat liver slices

Slices were cultured in medium containing glucagon (250 µg/ml final concentration) glucagon and dbcAMP (2 mM final concentration). Glucagon was disolved in the solvent supplied by the manufacturer. Sets of slices were at first cultured in normal media for the first 24 hours and then

transferred to media containing hormone for the next 24 hours and incubated at 37°C (Table 9). Glucagon appeared to decrease activity even in the presence of dbcAMP.

2.11 Effect of actinomycin D (40 μM) and dbcAMP (2 mM) on the activity of serine dehydratase in adult rat liver slices

Slices were cultured in normal medium for the first 24 hours at 37°C. Thereafter they were transferred to media containing actinomycin D (40 μM final concentration) and dbcAMP (2 mM final concentration) containing actinomycin D (40 μM final concentration). The samples were incubated for further 24 hours (Table 10). There appeared to be some diminution in the presence of actinomycin even with dbcAMP.

2.12 Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in liver slices from 13 day old rats over a 48 hour incubation

Young rats were used for experiments on the thirteenth day after birth. They weighed 20 - 22 gms and were killed by cervical dislocation.

Thirteen-day old rats were killed and livers quickly and carefully removed. Slices were incubated at 37°C. After 24 hours a number of slices were transferred to medium containing β-methasone (0.01 mM final concentration) and cultured for another 24 hours. The livers were assayed for SDH activity at zero time (in vivo), 24 hours, 48 hours as

TABLE 7

Effect of dibutyryl adenosine 3' :5'-cyclic monophosphate (1 mM, 2 mM) on the activity of serine dehydratase in slices of adult rat liver over a 48 hour incubation

SDH ACTIVITY: μmol NAD produced/min/gm liver			
Incubation time (hrs)	0	24	48
Control	2.58 ± 0.07	0.225 ± 0.045	0.105 ± 0.015
1 mM dbcAMP			0.173 ± 0.006
2 mM dbcAMP			0.162 ± 0.017

Adult rats were killed and the slices removed and cultured in normal medium for 24 hours. Some of the slices were assayed for enzyme activity while the rest were transferred to normal medium (control) or medium containing 1 mM or 2 mM nucleotide and incubated for a further 24 hours at 37°C. Cultures were removed after 48 hours and the slices assayed for enzyme activity. The atmosphere was renewed after 24 hours of incubation.

Each value is the mean ± S.E.M. of three observations. P values were calculated in order to find any statistical significance. With respect

to zero time control there was a significant decrease in enzyme activity on incubation at 24 hours $P < 0.01$ and 48 hours $P < 0.001$, while the decrease from 24 hours to 48 hours was not significant. On the addition of dbcAMP to the medium after an initial 24 hours incubation there was a significant induction of the enzyme by 1 mM dbcAMP ($P < 0.05$) while the induction by 2 mM dbcAMP though marked was not significant.

TABLE 8

Effect of dibutyryl adenosine 3' : 5'-cyclic monophosphate on the activity of serine dehydratase in slices of normal rat liver as a function of incubation time

SDH ACTIVITY: µmol NAD produced/min/gm liver					
Incubation time (hrs)	0	6	12	24	48
Slices Control	1.64 ± 0.02	0.18 ± 0.002	0.09 ± 0.003	0.05 ± 0.003	0.053 ± 0.001
Medium		0.103 ± 0.002	0.145 ± 0.002	0.12 ± 0.006	0.08 ± 0.003
Slices 2 mM dbcAMP		0.12 ± 0.003	0.12 ± 0.006	0.08 ± 0.002	0.045 ± 0.003
Medium		0.09 ± 0.003	0.12 ± 0.006	0.105 ± 0.003	0.145 ± 0.001

For details see next page

TABLE 8

Effect of dibutyryl adenosine 3' : 5'-cyclic monophosphate on the activity of serine dehydratase in slices of normal rat liver as a function of incubation time

SDH ACTIVITY: µmol NAD produced/min/gm liver					
Incubation time (hrs)	0	6	12	24	48
Slices Control	1.64 ± 0.02	0.18 ± 0.002	0.09 ± 0.003	0.05 ± 0.003	0.053 ± 0.001
Medium		0.103 ± 0.002	0.145 ± 0.002	0.12 ± 0.006	0.08 ± 0.003
Slices 2 mM dbcAMP		0.12 ± 0.003	0.12 ± 0.006	0.08 ± 0.002	0.045 ± 0.003
Medium		0.09 ± 0.003	0.12 ± 0.006	0.105 ± 0.003	0.145 ± 0.001

For details see next page

Normal rats maintained on a laboratory diet were killed and livers removed. The slices were cultured in normal medium (control) and medium containing dbcAMP (2 mM final concentration) and incubated for 48 hours at 37°C. Normal and experimental cultures were removed at 6, 12, 24 and 48 hours and the slices assayed for activity. The media of both control and experimental cultures were also assayed for enzyme activity. The atmosphere was renewed after 24 and 48 hours incubation.

Each value is the mean $\pm$ S.E.M. of three observations. P values were calculated in order to find any statistical significance. With respect to zero time activity, on incubation for 48 hours the activities were significantly lower $P < 0.001$. On addition of dbcAMP to the cultures and incubating for 48 hours there is an induction at 12 and 24 hours $P < 0.01$ respectively.

TABLE 9

Effect of dibutyryl adenosine 3' :5'-cyclic monophosphate (2 mM), glucagon (250 µg/ml) and glucagon plus dibutyryl adenosine 3' :5-cyclic monophosphate on the activity of serine dehydratase in fasted rat liver slices

SDH ACTIVITY: µmol NAD produced/min/gm liver			
Incubation time (hrs)	0	24	48
Control	3.41 ± 0.43	0.050* ± 0.004	0.053** ± 0.002
dbcAMP 2 mM		0.080*** ± 0.004	0.045 ± 0.003
Glucagon (250 µg/ml)			0.021*† ± 0.004
Glucagon (250 µg/ml) + dbcAMP (2 mM)			0.030*†† ± 0.003

For details see next page

Rats fasted for 24 hours prior to the commencement of experiments were killed and the livers removed. The slices were cultured in normal medium (control) or medium containing dbcAMP (2 mM final concentration) and incubated for 48 hours at 37°C. After 24 hours of incubation, control and experimental (dbcAMP) cultures were removed and the slices assayed for activity. Some of the control cultures were at this stage transferred to medium containing glucagon (250 µg/ml final concentration) and glucagon and dbcAMP (250 µg/ml and 2 mM final concentration respectively) and incubated for another 24 hours at 37°C. The glucagon was dissolved in the solvent provided by the manufacturer (Lilly Laboratories). After 48 hours the cultures were removed and the slices and medium assayed for activity. The atmosphere was renewed after 24 hours incubation.

Each value is the mean ± S.E.M. of four observations. * $P < 0.001$, ** $P < 0.001$ with respect to zero time control, *** $P < 0.001$ with respect to 48 hours dbcAMP. On comparing 48 hours dbcAMP to 48 hours control levels $P < 0.05$. *† $P < 0.001$ with repect to 48 hours control, *†† $P < 0.01$ with respect to 48 hours dbcAMP. Comparison of glucagon to glucagon plus dbcAMP showed no difference.

TABLE 10

Effect of actinomycin D (40 μM), dibutyryl adenosine 3' :5'-cyclic monophosphate (2 mM) and actinomycin D plus dibutyryl adenosine 3' :5'-cyclic monophosphate on the activity of serine dehydratase in normal rat liver slices

SDH ACTIVITY: μmol NAD produced/min/gm liver			
Incubation time (hrs)	0	24	48
Control	1.63 ± 0.07	0.050* ± 0.003	0.053** ± 0.002
2 mM dbcAMP		0.080*** ± 0.002	0.045 † ± 0.003
40 μM Actinomycin D			0.035 †† ± 0.003
Actinomycin D + dbcAMP			0.025 †† ± 0.002

For details see next page

Normal rats were killed and the livers removed. The slices were cultured in normal medium (control) or medium containing dbcAMP (2 mM final concentration) and were incubated for 24 and 48 hours at 37°C. After 24 hours of incubation samples of control and dbcAMP cultures were removed and assayed for serine dehydratase activity. Some of the control cultures at this stage were transferred to medium containing actinomycin D (40 μM final concentration) and dbcAMP containing actinomycin D (2 mM and 40 μM final concentration respectively). The cultures were incubated for a further 24 hours at 37°C. After 48 hours the cultures were removed and the slices were assayed for enzyme activity. The medium was also examined for serine dehydratase activity. The atmosphere was renewed after 24 hours incubation.

Each value is the mean $\pm$ S.E.M. of four observations. * $P < 0.001$, ** $P < 0.001$ with respect to zero time control, *** $P < 0.001$ with respect to 48 hours dbcAMP. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ with respect to 48 hours control, actinomycin D compared to 48 hours dbcAMP $P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ with respect to 48 hours dbcAMP, and actinomycin D plus dbcAMP compared to actinomycin D only $P < 0.02$.

control and a 24 hours sample containing the hormone as experimental. The results (Table 11) show as before a significant decrease in enzyme activity from zero time (*in vivo*) to 24 hours (*in vitro*). On comparing the *in vitro* results there is a decrease in activity from 24 hours to 48 hours, while the cultures containing β-methasone show induction over a period of 24 hours on comparing with the 24 hours control. These results are statistically significant.

2.13 Effect of dbcAMP (1 mM and 2 mM) on the activity of serine dehydratase in liver slices of 13 day old rats over a 48 hour incubation

Livers from 13-day old rats were cultured for 24 hours in normal medium at 37°C. Some of the slices were removed for 24 hours assay while the rest were transferred to normal medium as control, medium containing 1 mM and 2 mM dbcAMP as experimental samples (Table 12). There is approximately 40% decrease in activity from 24 hours to 48 hours incubation ($P < 0.001$). On comparison to the 48 hours control the induction by 1 mM dbcAMP was not significant while 2 mM dbcAMP induced the enzyme ($P < 0.05$).

2.14 Comparison of the activities of serine dehydratase in 13-day old and adult rat liver slices over a 48 hour incubation

Livers from 13-day old and adult rats were cultured in a medium containing foetal bovine serum for 24 hours. A few slices were assayed

for 24 hour activity while the rest were transferred to fresh medium for incubation for another 24 hours. The results obtained (Table 13) show that for a 24 hour incubation the level of SDH was twice as much in the 11-day old compared to the adult ($P < 0.001$). From 24 to 48 hours there was a drop in activity and similarly the 13 day old was higher than the adult.

3.0 Organ Culture of Rat Liver; Histological Studies

The organ culture system was examined for viability of the cells and hence suitability of studying the control of serine dehydratase *in vivo* and *in vitro* at 37°C.

3.1 Light Microscopy

Sections of a freshly excised normal adult rat liver (Fig. 19) were fixed in buffered formalin of neutral pH and stained with haematoxylin and eosin. The nuclei and cytoplasm have a normal appearance without any signs of degenerative changes.

Histological examination of slices of normal adult rat liver after 24 hours of incubation at 37°C reveals normal liver architecture of liver lobule with central vein and chords of cells radiating from it. There are no apparent degenerative changes in cells suggesting that the tissue is still viable (Fig. 20).

Figure 21 shows a section of a normal adult rat liver incubated for 48

TABLE 11

Effect of β-methasone (0.01 mM) on the activity of serine dehydratase in 13-day old rat liver slices

SDH ACTIVITY: μmol NAD produced/min/gm liver			
Incubation time (hrs)	0	24	48
Control	2.33 ± 0.13	0.24* ± 0.01	0.16** ± 0.01
0.01 mM β-methasone			0.26*** ± 0.03

13-day old rats maintained on a normal laboratory dietary regime were killed and the livers removed. The slices were cultured in normal medium and incubated for 24 hours at 37°C. A 24 hour sample was assayed for enzyme activity. Some of the samples were transferred to medium containing hormone (0.01 mM) and incubated for a fruther 24 hours at 37°C. The samples after 48 hours of incubation (control and experimental) were assayed for enzyme activity. A zero time (in vivo) activity was also recorded by assaying a sample of fresh liver.

Each value is the mean ± S.E.M. of nine observations * $P < 0.001$, ** $P < 0.001$ with respect to zero time control while *** $P < 0.01$ with respect to 48 hours control.

TABLE 12

Effect of incubation with dibutyryl adenosine 3' :5' -cyclic monophosphate on the activity of serine dehydratase in 13-day old rat liver slices

Time (hrs)	SDH ACTIVITY µmol NAD produced/min/gm liver	
24	Control	0.076 ± 0.005
48	Control	0.048 * ± 0.004
48	1 mM dbcAMP	0.060 † ± 0.005
48	2 mM dbcAMP	0.058 ** ± 0.003

13-day old rats maintained on a normal laboratory diet were killed and livers removed. The slices were cultured in normal medium and incubated at 37°C for 24 hours. A 24 hour sample was assayed for serine dehydratase activity while the others were transferred to medium containing 1 mM and 2 mM dbcAMP (final concentration) and incubated for a further 24 hours at 37°C.

Each value is the mean ±S.E.M. of nine observations, * $P < 0.001$, ** $P < 0.05$ with respect to 24 and 48 hours control respectively, while † with respect to 48 hour control was not significant. Also on comparing the levels of 1 mM to 2 mM dbcAMP they were found to be not significant.

TABLE 13

Comparison of the activity of serine dehydratase in 13-day old and adult rat liver slices

SDH ACTIVITY: µmol pyruvate produced/min/gm liver		
Time (hrs)	Adult	13-day old
24	0.12 ± 0.004	0.24 ± 0.004
48	0.087 ± 0.009	0.136 ± 0.012

Adult and 13-day old rats maintained on a normal laboratory diet were killed and the livers removed. The slices were cultured in normal medium containing foetal bovine serum and incubated for 24 hours and 48 hours at 37°C. Slices were removed at 24 and 48 hours and assayed for enzyme activity.

Each value is the mean ± S.E.M. of nine observations. Comparing 24 hour incubation to 48 hours for adult $P < 0.05$ and 13-day old $P < 0.001$. Similarly adult with respect to 13-day old for 24 hours $P < 0.001$ and 48 hours $P < 0.01$.

hours at 37°C. Pycnotic nuclei are visible. There are signs of cells undergoing autolysis. The organisation of chords is no longer regular and separation of the cells with degenerative signs of autolysis indicating some loss of viability of tissue.

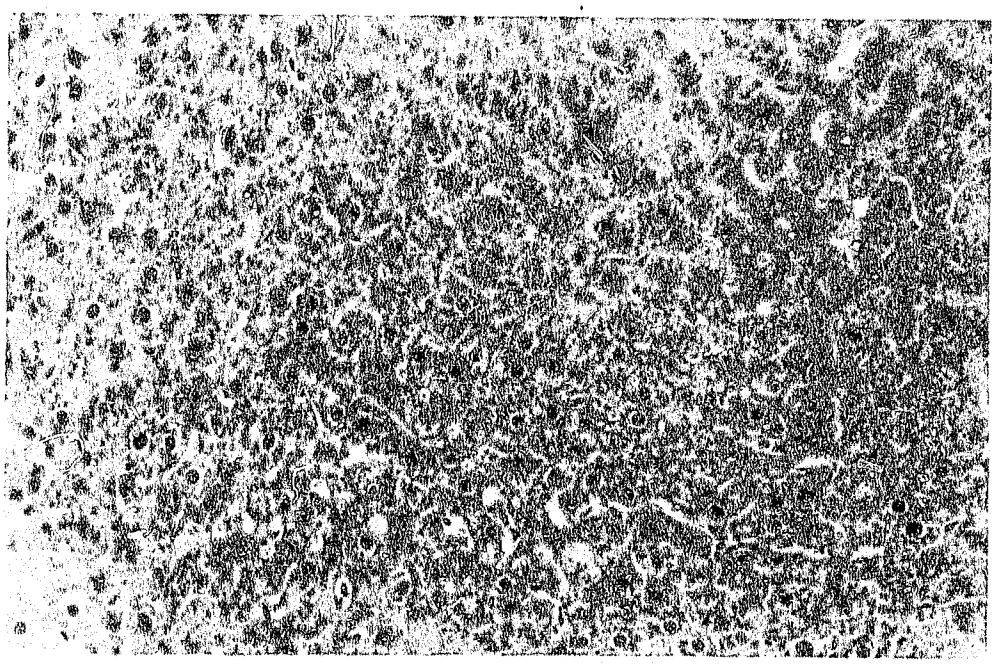
Figure 22 shows the histology of a normal adult rat liver incubated for 48 hours at 37°C, where there is complete karyolysis of nuclei (nucleus broken down). One cannot differentiate the cell membrane and normal architecture of the cell does not exist. The cell shows autolytic change by the loss of normal cell morphology. The changes are completely degenerative indicating complete loss of tissue viability.

3.2. Electron Microscopy

Rat liver is composed of polyhedral hepatic cells most of which have one or two nuclei. The tissue was fixed and stained as described in Materials and Methods and viewed using an AEI 801 transmission electron microscope.

In normal liver tissue freshly excised as in Figure 23 the hepatic nucleus contains a bilayered membrane as well as a nucleopore. The nucleus has chrom , and achromatin which is well established, mitochondria vary in shapes from round to oval with a double membrane. The inner membrane invaginates to form the cristae. Lipid inclusions and rough endoplasmic reticulum are scattered throughout the cytoplasm. The kupffer cells are of a reticulo-endothelial nature and the cells can perform phagocytic funtion. Lysosomes are present in the neighbourhood of bile canalicules

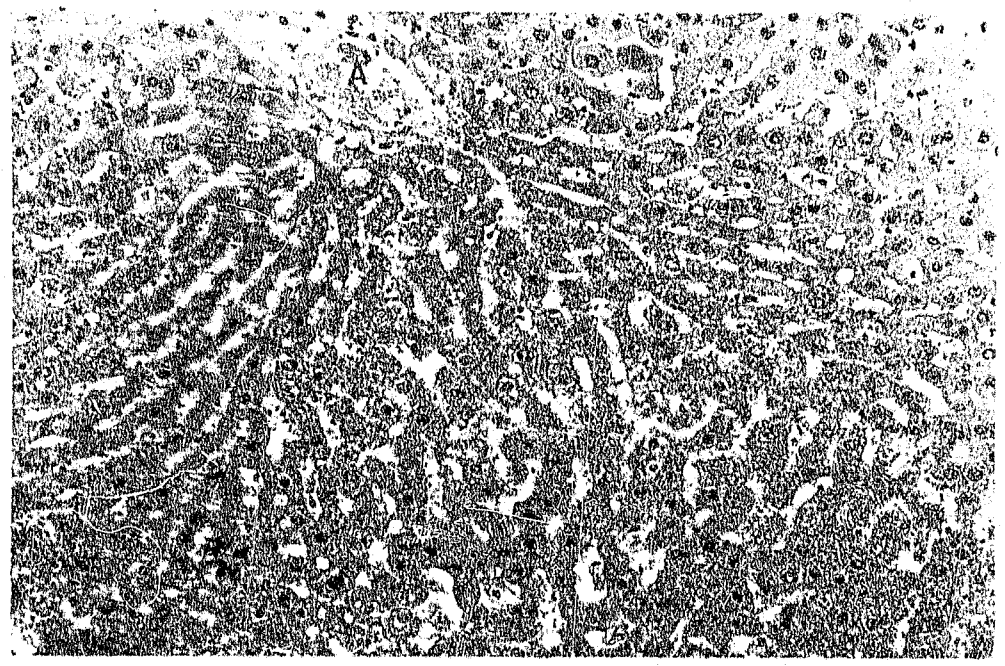
Figure 19 Slices of normal rat liver, freshly excised



The tissue was fixed in buffered formalin (neutral pH) and the sections stained with haematoxylin and eosin (see Materials and Methods). The tissue was viewed on a Reichert Univer photo microscope, with a green filter for contrast, showing normal histological characteristics.

(Magnification : 291X)

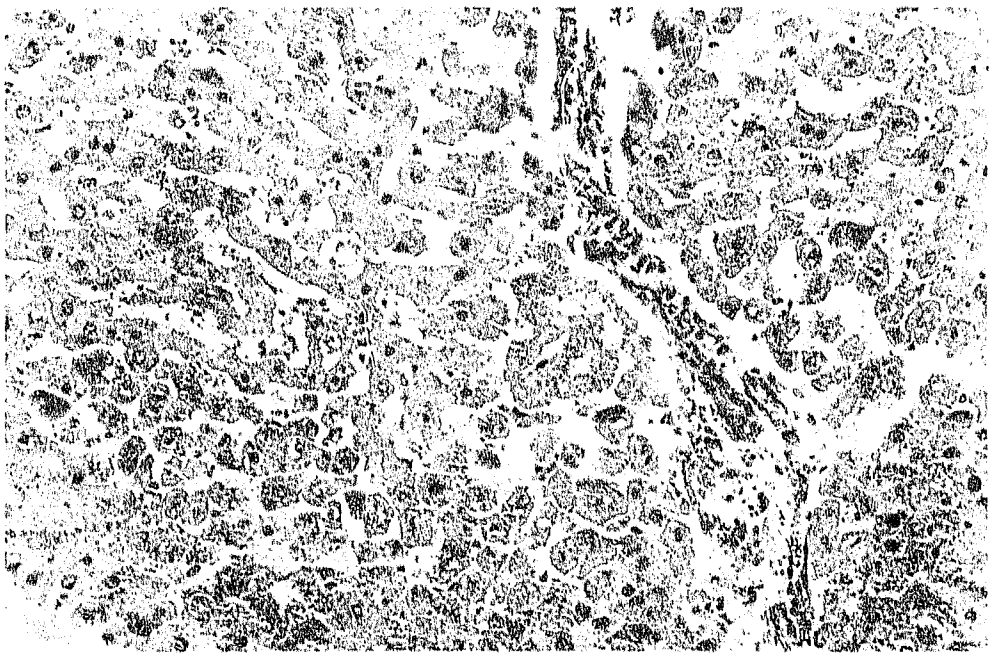
Figure 20 Slices of normal rat liver after 24 hours incubation at 37°C



Fixing, staining and viewing as in figure 19. The sections of the tissue viewed here shows the normal liver architecture of liver lobule (A) with central vein and chords radiating from it. The cells seem very viable and there are no apparent degenerative changes.

(Magnification: 291X)

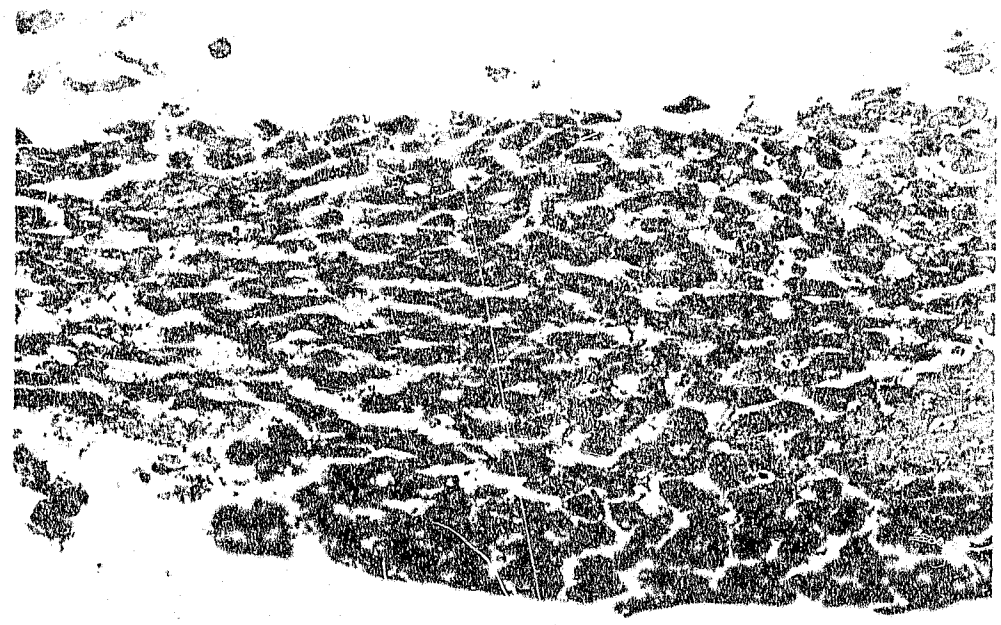
Figure 21 Slices of normal rat liver after 48 hours incubation at 37°C



The tissue was fixed, stained and veiwed as in figure 19. The section of tissue shown here shows that the cells are seperating with degenerative signs of autolysis and the organisation of the chords is no longer regular.

(Magnification: 291X)

Figure 22 Slices of normal rat liver after 48 hours incubation at 37°C



Fixing, staining and viewing as in figure 19. The sections of the tissue shown here, having been incubated for 48 hours at 37°C shows complete karyolysis of nuclei and the normal architecture of the cell has disappeared resulting in a completely necrosed cell.

(Magnification: 291X)

and the cell membrane is well established. Figure 24 shows a normal liver tissue after 24 hours of incubation at 37°C. The mitochondria and rough endoplasmic reticulum appear to be dilated. The mitochondria have also lost their inner cristae. The nuclear membrane is bilayered while the chromatin and achromatin are poorly preserved. Overall the tissue is still viable with little or no necrosis. In normal liver which has been incubated for 48 hours at 37°C (Figure 25) the mitochondria has an external membrane while the internal membrane is necrosed. Rough endoplasmic reticulum has lost its ribosomes. The nucleus has a double membrane with nucleopore while the chromatin and achromatin are poorly preserved and necrosed. The Golgi apparatus is also necrosed and the cell membrane is distorted.

Freshly excised liver from an adrenalectomised rat, fixed and stained under conditions similar to those described for normal rat liver shows glycogen to be present (FIGURE 26). The mitochondria have bilayered membrane while the inner cristae are poorly preserved. The nucleus has a double membrane and the chromatin and achromatin is condensed but well preserved. Microbodies are present in the cytoplasm and there is less rough endoplasmic reticulum. The lipids appear to be normal. The obvious difference between normal and adrenalectomised tissue is less endoplasmic reticular structure both smooth and granulated which probably accounts for the smaller cell volume observed in adrenalectomised cells. After incubating for 24 hours at 37°C the hepatic nuclear membrane seems

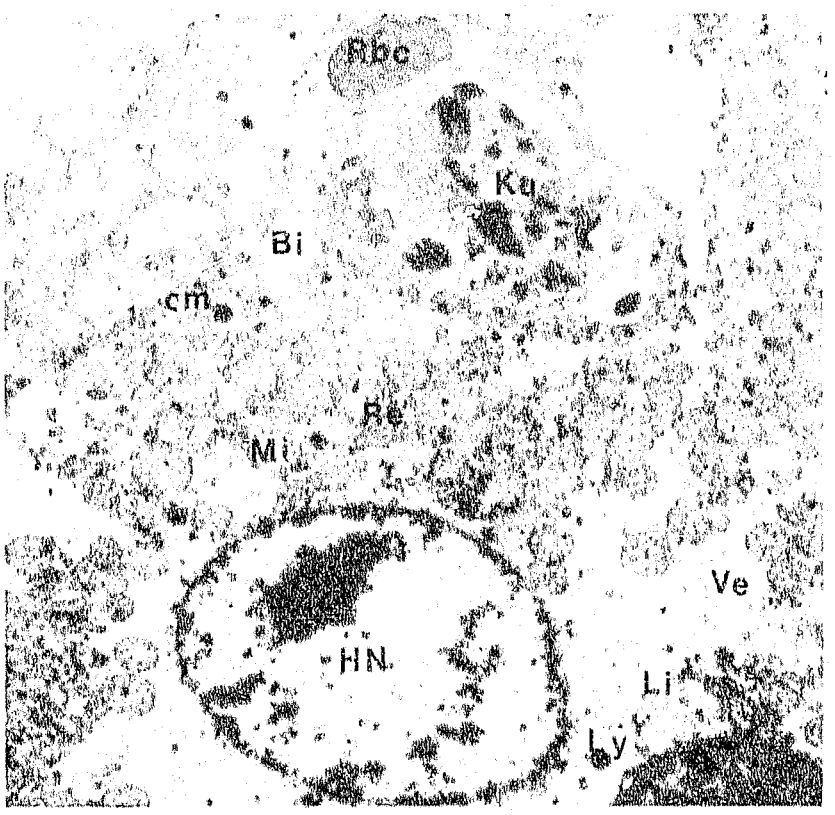
broken up although at certain places the double membrane is still visible. Chromatin and achromatin are still abundant but rough and granular. Glycogen forms into clusters and the mitochondria are distorted (Figure 27). Rough endoplasmic reticulum has lost some ribosomes and is poorly preserved or necrosed. Figure 28 shows results for the liver of an adrenalectomised rat incubated for 48 hours at 37°C. The nucleus has a double membrane with chromatin and achromatin scattered throughout. The cell membrane of bile canalicules is observed. The lysosome membrane is lost and most of the cytoplasmic organelles are poorly preserved, necrosed or lost as empty spaces occur.

3.3. Effect of hormones on the levels of potassium ions in normal and adrenalectomised rat liver slices as a function of incubation time

Samples of the freshly excised liver were assayed for potassium content (zero time samples). Liver slices were kept in M199 medium for approximately 20 to 25 minutes (time taken for the preparation of the organ culture) and some of them were assayed for potassium (this value was used as the zero time level for subsequent cultures), while the rest were cultured in normal medium, medium containing 2×10^{-6}M hydrocortisone and medium containing 0.01M β-methasone (Table 14).

In the freshly excised liver sample (normal rat) the level of potassium obtained is according to published values (≈ 70 μmol potassium/gm liver). The level of samples kept in M199 medium for approximately 20 to 25 minutes

Figure 23 Ultrastructure of a normal rat liver cell, freshly excised

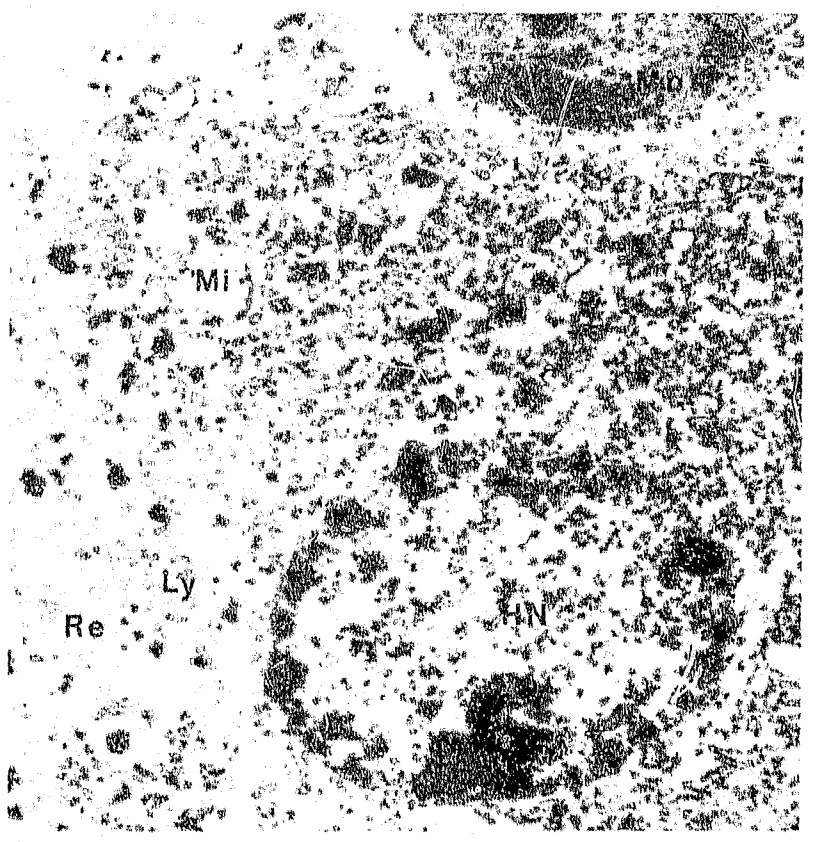


The freshly excised tissue was fixed in 3% glutaraldehyde-formalin solution buffered in Millonigs phosphate (pH 7.4) buffer, post-fixed in 1% osmium tetroxide embedded in an epon-araldite mixture. Sections were cut on a LKB ultramicrotome and the ultra thin sections (500-700 Å) were stained with uranyl acetate and lead citrate and viewed on an AEI 801 transmission electron microscope.

This section shows the hepatic nuclei (HN) surrounded by lysosomes (LY), lipids (Li), mitochondria (Mi) and rough endoplasmic reticulum (Re). On the top are some red blood cells (RBC) and Kupffer cells (Ku), alongside which are the bile canalicule (Bi) and a well defined cell membrane (Cm).

(Magnification: 5,600X)

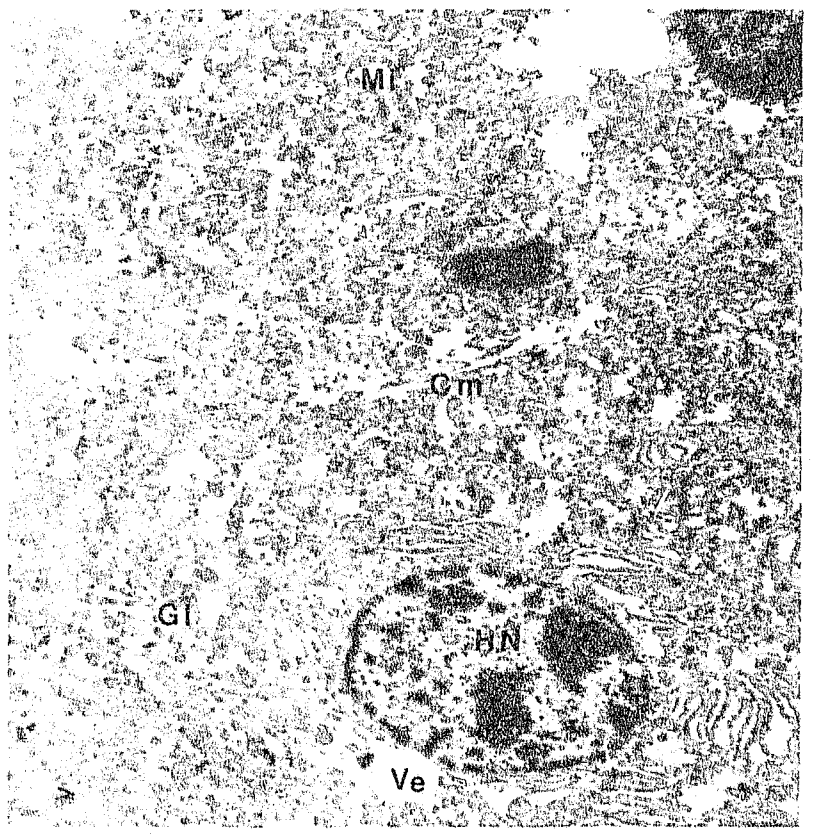
Figure 24 Ultrastructure of normal rat liver cell, after 24 hours incubation at 37°C



Fixing, embedding and staining as in figure 23 and sections viewed on an AEI 801 transmission electron microscope. The mitochondria (Mi), rough endoplasmic reticulum (Re) appear to be dilated. The nuclear membrane is bi-layered. Microbodies (Mb) and lysosomes (Ly) are present.

(Magnification: 8,750X)

Figure 25 Ultrastructure of normal rat liver cell after 48 hours incubation at 37°C

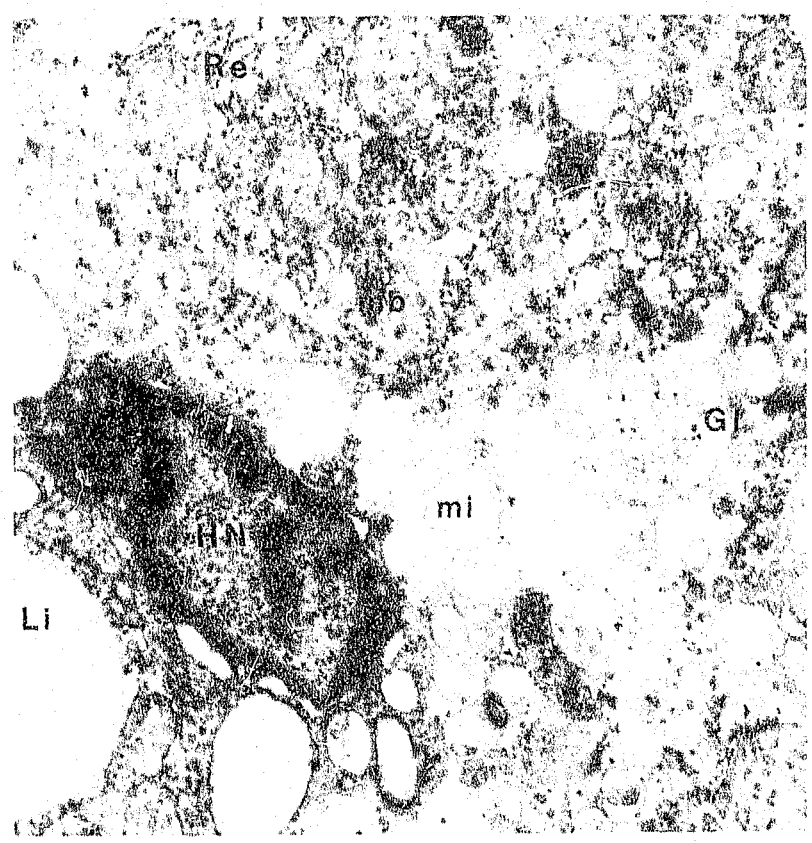


Fixing, embedding and staining as in figure 23 and viewed on an AEI 801 transmission electron microscope.

The tissue shows the hepatic nucleus has a double membrane with nucleopore. The mitochondria (Mi) has an external membrane while the internal membrane is necrosed. Rough endoplasmic reticulum (Re) has lost its ribosomes. The Golgi apparatus, achromatin and chromatin are poorly preserved and necrosed. The cell membrane (Cm) is distorted. Glycogen (Gl) is present.

(Magnification: 5,600X)

Figure 26 Ultrastructure of freshly excised adrenalectomised rat liver cell

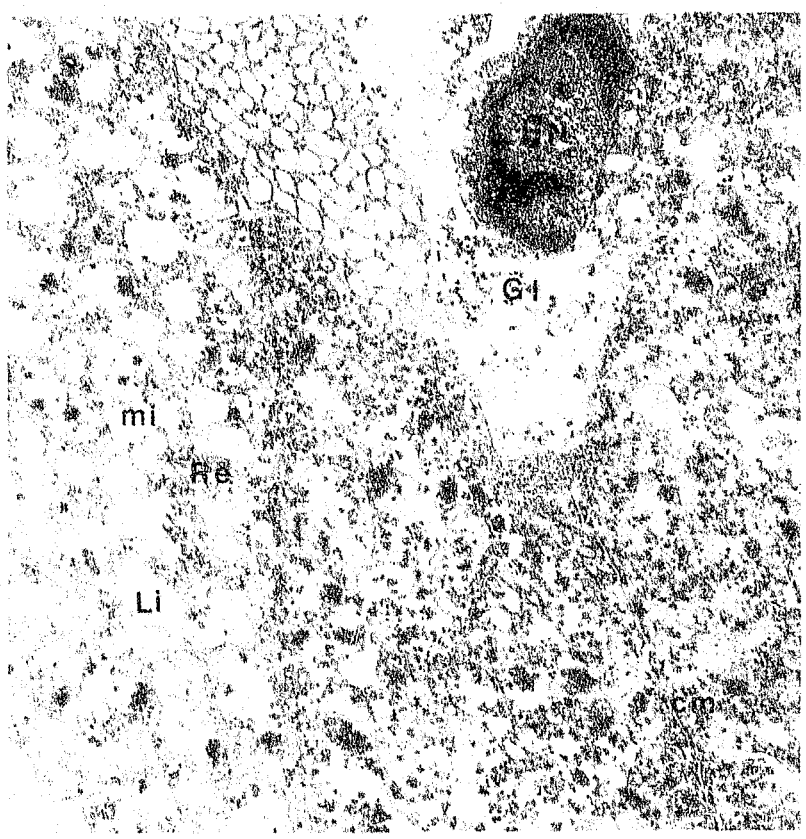


The tissue was treated as in figure 23 and viewed on an AEI 801 transmission electron microscope.

Glycogen (GL) is present, while the mitochondria (Mi) is a bi-layered membrane, the inner cristae of which are poorly preserved. The nucleus (HN) has a double membrane and the achromatin and chromatin are condensed but well preserved. Microbodies (Mb) are present in cytoplasm while there is less rough endoplasmic reticulum (Re). Lipids (Li) and lysosomes (Ly) are well preserved and appear to be normal.

(Magnification: 14,000X)

Figure 27 Ultrastructure of an adrenalectomised rat liver cell incubated for 24 hours at 37°C

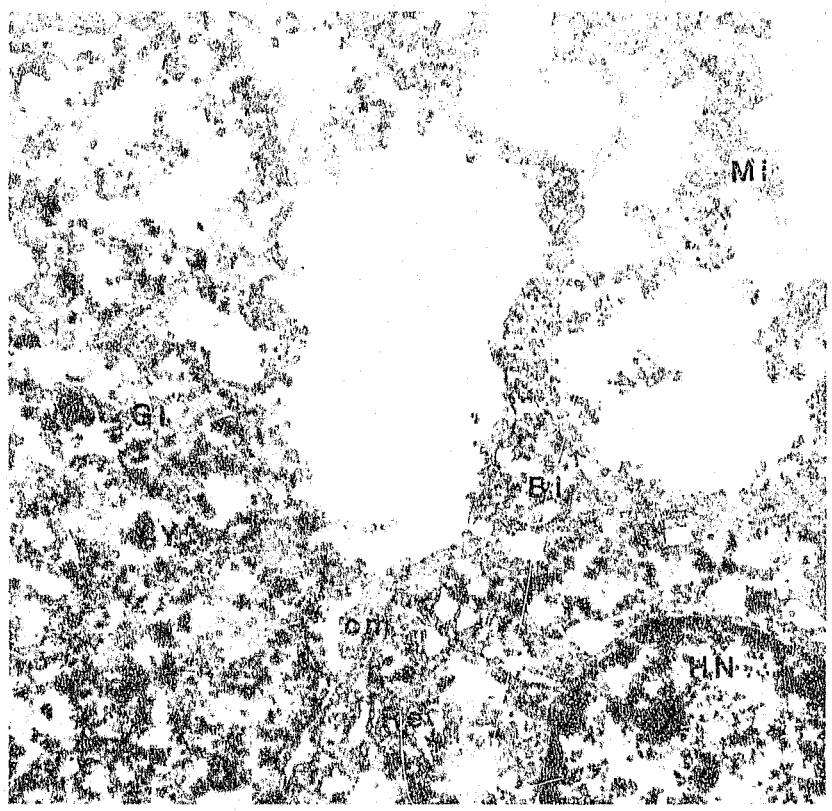


Fixing, embedding and staining as in figure 23 and viewed on an AEI 801 transmission electron microscope.

The glycogen (Gl) has formed into clusters, and the mitochondria (Mi) are distorted. The rough endoplasmic reticulum (Re) has lost its ribosomes and is necrosed. The hepatic nuclear membrane is broken up but at certain places the double membrane is still visible. Lipid (Li) and cell membrane (Cm) are present.

(Magnification: 5,600X)

Figure 28 Ultrastructure of an adrenalectomised rat liver cell incubated for 48 hours at 37°C



Fixing, embedding and staining as in figure 23 and viewed on an AEI 801 transmission electron microscope.

Most cytoplasmic organelle are poorly preserved, lost and necrosed as empty spaces occur. The lysosome membrane (Ly) is lost, the nucleus (HN) has a double membrane. Rough endoplasmic reticulum (Re), cell membrane (Cm) and cell membrane of bile canaliculus (Bi.) are present. Glycogen (Gl) is also present while the mitochondria (Mi) has half of its outer membrane left while the rest of the details are lost.

(Magnification: 8,750X)

drops to about 40 per cent ($P < 0.001$) to that of the sample assayed immediately. On further incubation at 37°C the levels remained constant for about one and a half hours whereafter there is a small increase ($P < 0.02$) in levels at 2 hours and then the levels drop back to pre-incubation with a slight decrease ($P < 0.05$) at 48 hours incubation.

In the freshly excised liver of adrenalectomised rats the level of potassium drops to approximately 30 per cent ($P < 0.001$) on standing in M199 medium for 20 to 25 minutes, after which there is a significant increase in potassium levels on incubation up to 2 hours ($P < 0.001$). There is a further increase at 4 hours ($P < 0.01$) thereafter the level drops to pre-incubation values up to 48 hours incubation.

Addition of 2 μM hydrocortisone or 0.01 M of β-methasone produced no clear change in the potassium content of the slices (Fig. 29).

TABLE 14

Effect of hydrocortisone (2×10^{-6}M) and β-methasone (0.01mM) on the concentration of potassium ions in normal and adrenalectomised rat liver slices as a function of incubation time

µmol potassium/gm liver				
Time	Normal	ADR	ADR + hydrocortisone	ADR + β-methasone
Zero In vivo	69.7 ± 2.3	71.5 ± 2.3		
Zero In vivo + Medium	27.6 ± 1.5	21.3 ± 1.9		
30 mins	30.8 ± 1.3	27.1 ± 0.2	27.0 ± 0.6	25.0 ± 0.8
1 hour	25.4 ± 0.6	28.9 ± 1.0	29.8 ± 1.9	25.6 ± 1.2
2 hour	34.2 ± 1.7	31.0 ± 1.5	28.9 ± 1.7	32.7 ± 3.3
3 hour	25.3 ± 0.4	23.8 ± 0.6	28.2 ± 0.3	29.7 ± 1.3
4 hour	25.2 ± 1.1	29.8 ± 1.9	22.5 ± 2.1	26.7 ± 0.3
24 hour	24.2 ± 0.9	25.4 ± 2.0	23.7 ± 0.8	23.4 ± 2.1
48 hour	23.7 ± 0.2	21.9 ± 0.6	14.8 ± 1.8	17.1 ± 0.5

ADR - Adrenalectomised

For details see next page

Normal and adrenalectomised rats were killed and their livers removed. The slices were cultured in normal medium, medium containing either 2×10^{-6} M hydrocortisone or 0.01 mM β-methasone (final concentrations) and incubated for 30 mins, 1,2,3,4, 24 and 48 hours respectively at 37°C. At the respective incubation times the slices were removed and assayed for potassium content as mentioned in Materials and Methods. For 24 and 48 hours the atmosphere was renewed at 12 and 24 hours respectively. Each value is the mean $\pm$ S.E.M. of nine observations. P values were calculated in order to establish the statistical significance of the results obtained.

The decrease in potassium concentration from normal zero time (freshly excised sample) to normal zero time in medium (kept in M199 medium for 20 - 25 mins) was significant $P < 0.001$. Comparing the significance of incubation on potassium concentration in normal rat liver slices, with respect to zero time (medium), for 30 minutes and 1 hour was not significant (N.S.) 2 hour $P < 0.02$, 3, 4 and 24 hours N.S. while 48 hours decreased $P < 0.05$. Decrease in zero time concentraion in adrenalectomised rat liver slices, to the same in medium was significant ($P < 0.001$). On incubation the concentration increased from zero time medium to 30 minutes $P < 0.01$, 1 hour $P < 0.01$, 2 hour $P < 0.001$, 3 hour N.S., 4 hour $P < 0.01$, 24 and 48 hours N.S.

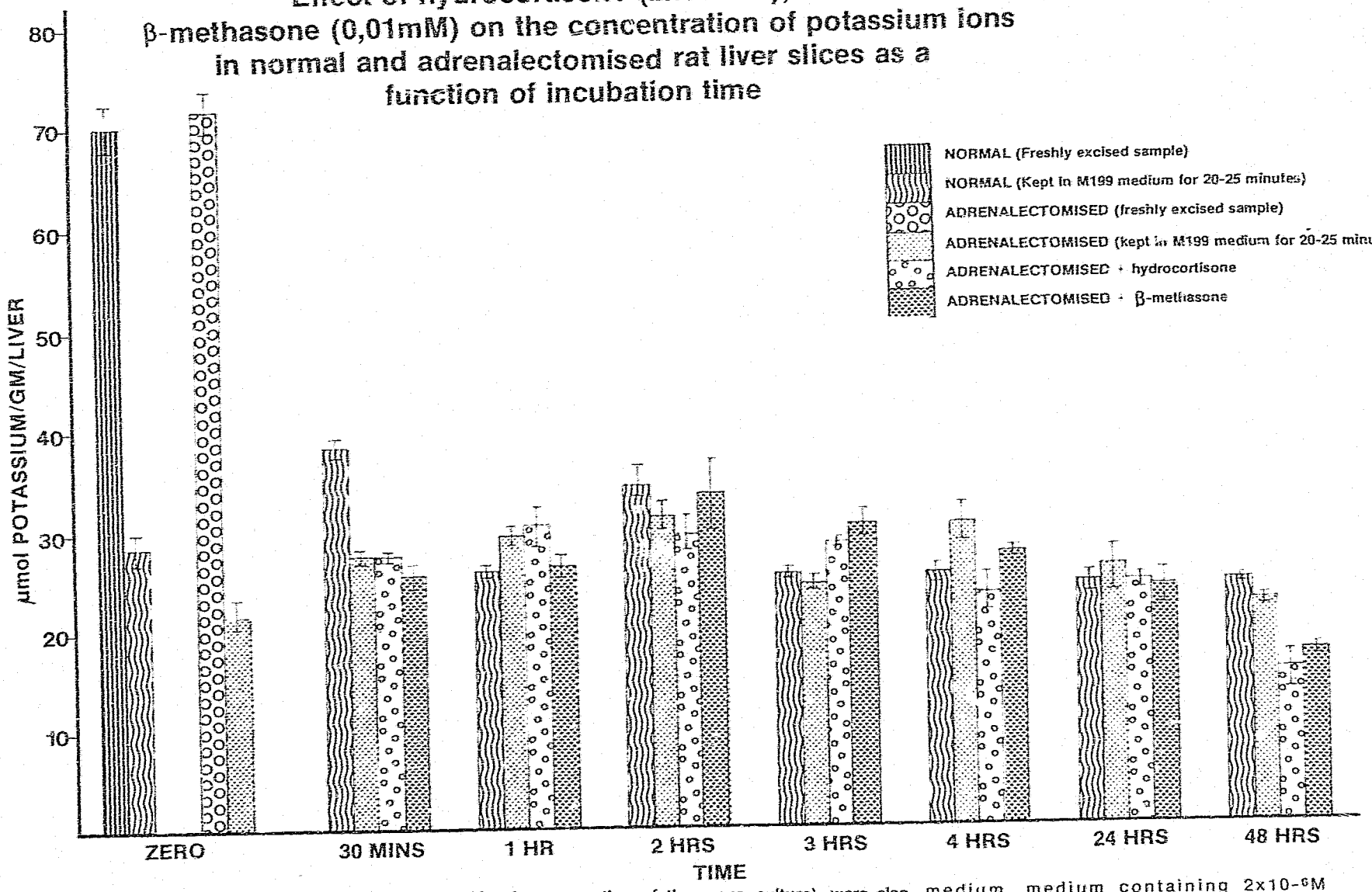
The effect of 2μmolar hydrocortisone (final concentration) on the concentration of potassium on incubation for 30 minutes, 1, 2 and 3 hours were all significant at $P < 0.01$, while at 4 and 24 hours they were N.S. and

there was significant decrease in concentration at 48 hours $P < 0.05$.

On addition of 0.01 mM β-methasone to the medium the concentration of potassium in the adrenalectomised rat liver slices on incubation at 30 minutes and 1 hour were N.S., 2 and 3 hours $P < 0.01$ respectively, 4 hours $P < 0.02$ while 24 hours were N.S. The marked decrease at 48 hours incubation was significant $P < 0.05$.

FIG. 29

Effect of hydrocortisone ($2x10^{-6}$ M), and β-methasone (0,01mM) on the concentration of potassium ions in normal and adrenalectomised rat liver slices as a function of incubation time



The potassium ion concentration was measured in freshly excised livers of normal and adrenalectomised rats (zero time). Liver slices kept in M199 medium for 20-25 minutes, (time required for preparation of the organ culture), were also assayed for their potassium content. This concentration was used as the zero time level for subseouent cultures. Liver slices cultured in normal medium, medium containing $2x10^{-6}$M hydrocortisone and medium containing 0.01 M β-methasone were incubated for various times at 37°C and assayed for potassium content.

IV DISCUSSION

I ASSAY METHODS

The colorimetric assay method for serine dehydratase of Suda and Nakagawa (1971) and the enzymatic method of Wimhurst and Manchester (1973) used to measure the levels of serine dehydratase and lactic dehydrogenase have been found to be suitable. The results *in vivo* obtained in this present study compare favourably with other published values. The levels of serine dehydratase determined by the colorimetric assay method compared favourably to those found by Rowsell *et al.* (1973) and also were similar to those found with the enzymatic method.

Enzyme activity was proportional to incubation time for atleast 30 minutes (figure 2) as also shown by Thorndike, (1972).

Since the levels of serine dehydratase activity recorded by assaying a normal rat liver using both the assay methods showed no significant difference, both these methods were used in different experiments.

2. ENZYME REGULATION

It is accepted that the activity of gluconeogenesis from amino acids (Wagle and Ashmore, 1961; Nadkarni *et al.*, 1960) in the liver is enhanced when the utilisation of glucose is limited under various hormonal and dietary conditions, such as diabetes, starvation or administration of carbohydrate free diet. Pyruvate is considered to be of great importance

as a carbon source for gluconeogenesis, since it is known that the sequence of gluconeogenic reaction is initiated by carboxylation of pyruvate to oxaloacetate (Henning et al., 1963; Wagle, 1963; Shrago et al., 1963). From this point of view, an important physiological role is suggested for serine dehydratase, which catalyses the degradation of serine to pyruvate and ammonia. The relation of serine dehydratase levels to gluconeogenic activities, however, is poorly understood. A study of the hormonal and dietary control of serine dehydratase was carried out in vivo and in vitro in rat liver.

3. IN VIVO STUDIES

Serine dehydratase activity in rat liver has been shown to be regulated by the antagonistic actions of insulin and glucocorticoids in alloxan induced diabetic rats by Ishikawa et al. (1965) and later in normal rats by Freedland et al. (1966). At the same time, however, Ishikawa et al. (1965) also reported that glucocorticoids might be essential for the increased activity in insulin deficiency. The present study (table 2) has shown that the enzyme activity increased markedly on overnight starvation, in agreement with the findings of Suda (1967). Adrenalectomy resulted in a slight increase in fed animals (C'Sanyi and Greengard, 1967) while on starvation and adrenalectomy there was no effect. The change in enzyme level in starved rats is noteworthy since starvation may be one of the situations in which it is demonstrated how the hormonal control mechanism

is working in the animal (Ishikawa et al., 1965). The results obtained conform with those shown by Ishikawa and Nakajima (1970) and by Suda (1967). The injection of 3 mg hydrocortisone did not raise the enzyme activity in the starved animals whereas a significant increase was observed in the fed ones. Ishikawa and Nakajima (1970) found that injections of hydrocortisone to adrenalectomised fed rats increased the enzyme activity only slightly while in the starved animals the increase was nearly two fold.

On examining the effect of hydrocortisone on the levels of serine dehydratase, Jost et al. (1970) found no stimulation of the enzyme when rats were treated with 2 mg hydrocortisone per 100 gm body weight. Fenaino (1967) found that cortisone was not necessary for the induction of serine dehydratase, but it greatly enhances the enzyme activity in the presence of amino acids. Hurvitz and Freedland (1968) and Pitot et al. (1965) in their study of the interrelationships of the action of dietary protein and hydrocortisone on adaptive enzymes of amino acid metabolism in rat liver have shown that serine dehydratase was the only enzyme which did not respond to cortisone in the absence of dietary casein. In the presence of 90% dietary casein, serine dehydratase activity was enhanced by hydrocortisone due to an increased rate of its synthesis. Ishikawa et al. (1965) found that on the administration of hydrocortisone serine dehydratase was induced significantly in the livers of alloxan diabetic, adrenalectomised rats whereas little or no induction occurred in the livers

of adrenalectomised rats with intact Islets of Langerhans. Conversely, Fallon et al. (1966) found a marked increase in the enzyme levels when cortisone was administered to rats fed laboratory chow or 2% casein diet.

Fig. 8 shows the results of hydrocortisone in rats previously adrenalectomised and thyroidectomised (double operations). There was no induction of the enzyme by the hormone. On the addition of triiodothyronine to rats doubly operated, there was a significant induction of the enzyme (Fig. 9) Ishikawa et al. (1965) showed that administration of triiodothyronine for 5 days caused increase in the activity of the enzyme in adrenalectomised rats. Freedland (1964) also reported increase in enzyme activity on the administration of thyroxine. The present studies also showed that the simultaneous injections of hydrocortisone and triiodothyronine to doubly operated rats resulted in an additive response with significantly increased activity of the enzyme (Fig. 10).

In lactic dehydrogenase studies a slight increase in enzyme levels on starvation and adrenalectomy was observed (Table 2).

Addition of hydrocortisone to adrenalectomised fed animals resulted in an increase in the enzyme levels while in the adrenalectomised starved animals the addition of hormone showed a decrease in the enzyme activity. These findings are in partial agreement with that found by Fallon et al. (1966) who stated that no significant changes in lactic dehydrogenase enzyme activity occurred when cortisone was administered for 7 days to animals fed Purina laboratory chow.

From the results obtained it seems that hydrocortisone has no significant inducing effect on the levels of serine dehydratase and lactic dehydrogenase. This is substantiated by other workers as mentioned earlier; however diet does seem to play an important regulatory role.

4. IN VITRO STUDIES

Serine dehydratase and lactic dehydrogenase activities fell very markedly on incubation of slices at 37°C. Slices from adult rat were initially incubated for 24 hours. There was a significant decrease in enzyme level from the initiation of these experiments (*in vivo*) to 4 hours of incubation and a further decrease at 12 hours, whereafter the levels remained fairly constant (Fig. 11 and 13). Addition of serine was without effect and the decrease in enzyme activity was the same as in experiments without substrate (Fig. 12). No serine dehydratase activity was found in the medium. However activity of lactic dehydrogenase in the medium steadily increased with incubation time reaching a maximum at 12 hours followed by a decrease at 24 hours. This however was still well above zero initial levels, indicating that the enzyme has leaked out of the tissue (Fig. 14). MacDonnel *et al.* (1975) also found that liver explants from 20 day old foetuses, cultured for 48 hours in the absence of serum, released 70% of their total soluble protein in the medium. In the presence of serum this loss still amounted to 60%. The concentration of total particulate protein remained unchanged but there was some translocation of mitochondrial enzymes to the cytosol and enzymes expected to increase at this stage of development

failed to do so. Goodridge et al. (1974) reported similar findings showing that lactic dehydrogenase was released into the medium.

The addition of hydrocortisone to the medium in cultures of normal and adrenalectomised rat liver slices did not have any significant inducing effect on the levels of serine dehydratase. Instead there was a significant decrease in the levels of the enzyme on comparison to zero time control levels (Fig. 15 and 16).

The addition of β-methasone to the medium of normal and adrenalectomised rat liver cultures also did not have any significant inducing effect on the levels of serine dehydratase (Fig. 17 and 18). As in hydrocortisone the decrease in serine dehydratase activity is probably due to the fact that the liver is deprived of the supply of endogenous glucocorticoids and not to any other effect associated with maintenance in culture.

Serine dehydratase activity decreased on adrenalectomy and starvation while activity in the adrenalectomised fed cultures increase. Addition of hydrocortisone to the culture medium resulted in a decrease in enzyme levels in the adrenalectomised fed samples and an increase in the adrenalectomised starved (Table 3). However in view of the obvious big loss of enzyme from the tissue and the very low activities observed in the slices it is questionable whether any physiological meaning can be attached to these changes.

Wicks (1968) in studies on transaminase activity showed that the addition

of hydrocortisone to fresh explants of foetal liver produced a substantial increase in tyrosine transaminase activity after a lag of 6 to 12 hours. The increase in enzyme activity continued throughout the culture period. When hormone was added to 42 hour cultures of liver from term foetuses, there was only a brief lag before enzyme activity increased as in the case with the adult (Kenney and Kull, 1963). Wicks (1968) found that the continual increase in transaminase activity in foetal liver organ cultures stands in contrast to the response observed in adult rats. In the latter case, the induced transaminase activity falls back to the control level within 10 to 12 hours after hormone is given (Garren et al., 1964). One obvious explanation for this difference is the absence of a system in the organ cultures for the elimination of hydrocortisone and its metabolites. Kenney and Flora (1961) have shown that this steroid is cleared rapidly from the liver in adult rats.

In the present studies adult and 13 day old rat livers were cultured for 48 hours at 37°C. Over a 24 hour incubation period, serine dehydratase activity was twice as much in 13 day old animals as in adult. After 48 hours incubation serine dehydratase activity was still higher in the youger animal (Table 13). The addition of β-methasone or dibutyryl cyclic AMP to the medium after 24 hours and incubated up to 48 hours induced serine dehydratase levels in 13 days old rats. Normal and adrenalectomised adult rat liver slices, cultured in medium con-

taining hydrocortisone or β-methasone and incubated for 48 hours, did not induce serine dehydratase activity (Table 4 and 6).

No induction by hydrocortisone was observed even after a further incubation for another 10 hours (after the initial 48 hour incubation). Instead the hormone appeared to decrease the enzyme levels on comparison to zero time control levels. In the normal rat liver cultures containing β-methasone small induction occurred at 6 and 8 hours after the intial 48 hours incubation. Some enzyme activity was recorded in the medium indicating that the enzyme had leaked out and as with adult slices it is difficult to draw conclusions from these results. The mechanism responsible for such hormonal effects on enzyme levels is not clearly established but the lack of an ir vitro effect of hydrocortisone suggests an indirect mechanism.

Serine dehydratase is induced by dibutyryl cyclic AMP as shown in table 8. Over a 48 hour incubation period, dibutyryl cyclic AMP significantly induced the enzyme levels in adult rats at 12 and 24 hour incubation. On futher incubation (after an initial 48 hours) the enzyme was induced at 8 hours. As mentioned earlier dibutyryl cyclic AMP significantly induced serine dehydratase activity in 13 day old rat liver slices incubated for 48 hours. Jost et al. (1970) found that of all the cyclic nucleotides and adenosine monophosphates tested only cyclic 3`, 5`- AMP (or its dibutyryl derivative) induced rat liver serine de-

hydratase *in vivo* in agreement with the *in vitro* findings presented in this study.

Injections of glucagon or an oral intubation of casein hydrolysate increases the activity of rat liver serine dehydratase (Pitot and Peraino, 1964; Peraino and Pitot, 1964; and Peraino *et al.*, 1965). As mentioned earlier dibutyryl cyclic AMP induces the levels of serine dehydratase *in vitro* in adult and 13 day old rat liver slices. The addition of glucagon to the medium resulted in a decrease in enzyme activity compared to control levels; however if dibutyryl cyclic AMP is added to the medium containing glucagon and followed by further incubation for 48 hours there was a one third increase in the levels of serine dehydratase compared to the effect of glucagon alone (Table 9), suggesting that dibutyryl cyclic AMP is an intermediate in the induction of serine dehydratase by glucagon. Similar findings have been described by Jost *et al.* (1970) *in vivo*.

Addition of actinomycin D to the medium containing adult rat liver slices and incubation for 24 hours resulted in a 33% inhibition of serine dehydratase activity compared to control levels (Table 10). On addition of dibutyryl cyclic AMP to the medium containing inhibitor, the enzyme activity was futher inhibited (40%). It is generally accepted that actinomycin D inhibits DNA-dependent RNA synthesis (Goldberg and Rabinowitz, 1962; Revel *et al.*, 1964). It has also been

reported that it interferes with the passage of newly formed RNA from the nucleus to the cytoplasm (Georgiev et al., 1963; Girard et al., 1964; Georgviev, 1967). Actinomycin D inhibited the induction of serine dehydratase formation by dibutyryl cyclic AMP, Suggesting that the cyclic nucleotide exerted its effect either at the level of transcription or transfer of the message to the cytoplasm (Jost et al., 1970). Wicks (1968) found that the addition of actinomycin D inhibited induction of tyrosine transaminase and there was a direct relationship between the degree of inhibition of RNA synthesis and enzyme induction after about 20% inhibition of RNA synthesis had occurred. He found that large doses of the antibiotic, about 25 µg/ml, were necessary to achieve 90 to 95% inhibition of induction in organ culture. This is as much actinomycin in each dish as is required to inhibit enzyme induction completely in a 150 gm adult rat (Greengard et al., 1963). Apparently there is some difficulty in the entrance of the inhibitor into the explants, but it is also possible that the inhibition of enzyme induction resulted from a general toxic effect and not from an inhibition of RNA synthesis. In the present studies a final concentration of 40 µM actinomycin D was used which was far below the concentrations used by Wicks (1968) and Greengard et al. (1963), therefore inhibition by toxicity is excluded.

5. ORGAN CULTURE OF RAT LIVER

The organ culture system as described in Materials and Methods was

able to maintain liver tissue in a viable state for at least 24 hours. This was shown by criteria of light microscopy, electron microscopy and the estimation of potassium ions. The viable layer of the slices (that which is in contact with oxygen, 0.2 mm thick) correlates with that found by Trowell (1959) and Campbell and Hales (1971). The reason for the presence of basophilic cells is not know but they were absent in liver slices from adrenalectomised rats after 24 hours in organ culture. This liver also retained its relatively smaller cell volume under culture and a layer of viable tissue similar to that seen in normal rat liver was present, indicating that this liver survived as well as normal rat liver.

Under electron microscopy some of the cells appeared to have undergone some change in structure. Dilation of mitochondria would indicate depletion of cellular ATP, but most of the mitochondria appeared normal. Although some of the endoplasmic reticulum lost its ribosomes most appeared normal. Cells in slices from adrenalectomised rats responded in the same way which indicated the feasibility of using this liver in organ culture.

Majno (1963) found that depriving liver of oxygen for 45 minutes resulted in the majority of cells in the organ dying, which indicates that changes in liver cells resulting in death occuring at an early stage. It was for this reason that the preparation of the organ culture in this present study was completed within 30 minutes of the animal being

killed.

About 50 to 100 mg tissue could be maintained in a viable state for at least 24 hours using the organ culture method described. These quantities were the minimum amounts of liver that could be used in assaying serine dehydratase. The chief disadvantage of the method of Campbell and Hales (1971) was that only 10 mg of liver could be cultured per dish. The requirement for a high oxygen atmosphere was similar to that of Campbell and Hales as well as Laufs and Walker (1970). The former workers experimented with higher oxygen pressures but these resulted in rapid tissue death of cultures. That the cultures needed a high serum content was supported by the results of Lurie et al. (1969) who found a higher serum content medium more suitable for maintaining adult mouse liver in organ culture.

Since this work was started, several groups of workers have developed methods for the isolation and maintenance of non-dividing parenchymal adult rat liver cells in culture. Usually it was necessary to maintain the cells on a collagen support and the medium required a protein supplement in the form of albumin and also insulin (Michalopoulis and Pitot, 1975; Pariza et al., 1975). Michalopoulis and Pitot (1975) maintained liver cells in a viable state for 3 weeks. A limitation of this cell culture method was that it was applicable only to older animals since it involves perfusion of the liver, while the present method was

applicable to mammalian livers of all ages. The reason for the insulin requirements by the cell cultures and the high serum concentration by the organ cultures could be one and the same. Hoffman et al. (1973) isolated protein componenets from serum with insulin-like properties and these could stimulate cell growth of rat fibroblasts. The albumin requirement of the cell cultures would be met by the high serum concentrations as well, although the albumin content of the cell culture medium was considerably lower than that of serum (White et al., 1968). The reason for the necessity of whole egg ultrafiltrate in the organ culture medium could be the presence of insulin-like molecules in the egg extract. As components with a molecular weight greater than 12,000 are excluded in the filtrate (Wolf and Quimby, 1964), molecules similar to insulin (m.wt 5,700, White et al., 1968) might easily be included in the ultrafiltrate. In some experiments the ultrafiltrate was replaced by foetal calf serum.

The finding that preincubation of the slices improved the response is similar to that of Wicks (1968) who discovered that preincubation of foetal liver organ culture explants shortened the lag period before induction. The difference in the technique used by Wicks (1968) to that used in this study is that in the former method the explants were supported directly in the medium while in the latter the slices were supported above the culture medium. Furthermore the differences in the

two types of liver used, adult liver parenchymal cells being more tightly packed with less sinusoidal space than foetal liver, implies that diffusion could take place more easily in the relatively greater fluid volume surrounding the foetal hepatocytes. The shrinkage of the cells in adrenalectomised rat liver might also have resulted in larger sinusoidal volume - and this too may partly account for the quantitative difference in the induction between normal liver and liver from adrenalectomised rats.

It is known that slices of rat liver immersed in nutrient media for one or two hours release a considerable portion of their soluble protein content (Streffer and Williamson, 1965). These workers also found that the presence of horse serum in the medium decreased significantly the loss of soluble protein from the explants, but even so, only about 30% of the total was still present in the explants at 48 hours. The addition of insulin, cortisol and glucagon to the medium, separately or in various combinations, did not exert any significant influence on the protein content of the explants (MacDonnel et al., 1975). It was suggested that the possible loss of some protein from foetal liver explants may be attributable to the elimination of haematopoietic cells (Wicks, 1968; Kirby and Hahn, 1973). However, according to cytomorphometric analyses (Greengard et al., 1972), haematopoietic tissue accounts for only 38% and 25% of the liver mass at 5 and 2 days before birth respectively, so

that even a complete expulsion of these cells would not account for the loss of over 60% of the total soluble protein content observed. Thus, MacDonnel et al. (1975) attributed the loss of soluble protein to leakage from the hepatocytes.

The definition of 'viability' is, however, a major problem. For example explants are capable of incorporating precursors into protein and RNA, (Wicks, 1968; Sereni and Sereni, 1970; Räihä et al., 1971) but these synthetic capacities have not been quantitatively compared with those obtained in vivo. The preferential accumulation or loss of particular enzymes that are known to increase or diminish during this period in vivo may provide more specific criteria for continued differentiation. The only study (Tourian, 1973) that attempted this type of comparison yielded a negative result. Previous work done on liver slices (Geyer et al., 1956; Flink et al., 1960; Parsons and Von Rossum, 1962; Judah and McLean, 1962) and perfused liver (D'Silva and Neil, 1954) found that an early event preceeding necrosis was a change in the ion content of the tissue. Possible causes of the ionic changes were either membrane damage or loss of sodium pump activity or both. The criteria employed so far to ascertain the viability of the organ culture system used have been histological (light and electron microscopy). Since on a 24 hour incubation at 37°C there were signs of some structural degeneration, which at 48 hours incubation led to poorly preserved cell organelles, the level of potassium ions was estimated in normal and adrenalectomised rat liver

liver slices over a 48 hour incubation at 37°C.

The level of potassium in a freshly excised normal or adrenalectomised rat liver agreed with known published values (≃ 70 µmol K/gm liver). On standing in M199 medium for approximately 20 to 2[illegible] minutes, the levels dropped to about 40% of the control, after which there was a slight increase in potassium levels at 2 to 4 hours incubation. On additional incubation they dropped to pre-incubation levels and remained fairly constant. The addition of steroids (hydrocortisone and β-methasone) to the medium of liver slices from adrenalectomised rats followed by incubation for 48 hours at 37°C, showed that the levels of potassium followed the same pattern as that of normal or adrenalectomised samples up to 24 hours. From 24 to 48 hours incubation there was a significant drop in potassium levels (Table 14).

It seems reasonable to ascribe the initial fall of liver potassium concentration to the injury caused by slicing and the anoxia lasting from the time of cessation of circulation in the animal to the immersion of the slices in fluid equilibrated with oxygen and carbon dioxide. Flink et al. (1960) suggested that anoxia and trauma are important factors from their experiments in which the potassium loss was greatest when the time of preparation was the longest. They also demonstrated that on anaerobic incubation, anoxia was indeed an important cause of the initial loss of potassium. Houssay et al. (1973) observed a rise in serum potassium in the intact animal which had been subjected to asphyxia

and the excess potassium has been shown to arise chiefly from the liver. Dixon (1949) observed no loss of potassium from brain slices if the medium contained glucose in spite of anaerobic incubation. Flink et al. (1960) showed that although about 70% of intracellular potassium of the rat liver is quite labile and passes rapidly into extracellular fluids when the liver tissue is subjected to injury and anoxia, it is capable of being largely restored to its normal intracellular concentration if aerobic conditions are resumed and if a suitable concentration of potassium is maintained in the extracellular fluid. Therefore the drop in potassium from control to pre-incubation levels can be explained by the initial injury caused by slicing of the liver and of anoxia while it is kept in medium for approximately 20 to 25 minutes before incubation. As shown in the results (Fig. 29) incubation for the first 2 to 4 hours resulted in a rise in potassium levels.

From these potassium results it can be said that the viability of the slices in the culture system used here appear to be maintained.

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