

SOME ENZYMES OF DNA SYNTHESIS
IN NORMAL AND CANCEROUS HUMAN AND RAT LIVERS.

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DECLARATION

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

SIGNED:

R. R. Cummings

ABSTRACT

DNA polymerase, thymidine kinase, thymidylate kinase, thymidylate synthetase and deoxycytidylate deaminase have been measured in rat and human normal livers and hepatomas, in human developing liver, and in cell-lines derived from human and rat hepatoma tissues. These key enzymes of DNA synthesis showed increased levels in a 3'-methyl-4-dimethylaminoazobenzene-induced rat transplantable hepatoma, to a degree which corresponded with a rapid tumour growth-rate. The levels of thymidylate synthetase, thymidylate kinase, deoxycytidylate deaminase and DNA polymerase were increased in human hepatoma, suggesting an increase in the rate of DNA synthesis. The increases in activity of these enzymes in human hepatoma were small in comparison with those in the rat hepatomas, suggesting that the growth-rate of human liver tumours is relatively slow. Thymidine kinase levels in human normal livers and hepatomas were similar. Very high levels of deoxycytidylate deaminase were measured in human normal liver. Deoxycytidylate deaminases from both human and rat normal and cancerous livers were able to use 5-methyl-deoxycytidylate as a substrate to a similar or greater extent than deoxycytidylate, suggesting a possible alternative pathway of deoxythymidylate production. A slow-growing human hepatoma cell-line exhibited enzyme levels which were similar to those in human hepatoma tissue. On the other hand, rapidly-growing cell-lines from both the rat and human hepatomas generally showed higher levels of activity for the enzymes assayed than did the corresponding rat and human tissues. Levels of these enzymes in human foetal liver appeared to vary as a function of gestational age, with maximum activity of all five enzymes occurring between 17 and 19 weeks gestation.

Thymidine kinase was partially purified from human normal liver

and hepatoma tissues using ion-exchange chromatography on DEAE-cellulose, phosphocellulose and CM Sephadex; and gel-filtration on Biogel A1.5. Four forms of the enzyme were purified from each tissue. These were designated thymidine kinases IA, IB, IIA, and IIB. The relative proportions of the corresponding enzyme forms from normal and cancerous liver were similar, with IIB being the major form.

Some physical and kinetic properties of the thymidine kinase enzyme forms were studied. The molecular weights and pH optima of each of the four thymidine kinase forms were different. On heating at 55°C, all of the enzyme forms showed activity loss which could be reduced by the presence of thymidine. The thymidine kinase forms differed with respect to their Michaelis constants for thymidine. All forms of the enzyme used ATP and CTP, but not dATP or dCTP, as phosphate donors; and were inhibited by dCTP, dTMP, dTDP, dTTP, and dATP. Phosphate acceptor studies showed that thymidine, deoxyuridine, deoxycytidine and 5-fluoro-deoxyuridine were effective substrates for human liver thymidine kinases. The presence of the thymidine analogues deoxycytidine, 5-fluoro-, 5-bromo-, and 5-iodo-deoxyuridine inhibited phosphorylation of the thymidine kinase forms, with the exception of the IA fraction from human hepatoma which was activated in the presence of 5-fluoro-deoxyuridine. The specific activities of thymidine kinases in human hepatoma resembled those in human normal liver, and there was little difference between the physical and kinetic properties of the corresponding thymidine kinase forms present in human normal and cancerous liver, suggesting that there are no tumour-specific forms of the enzyme in the human hepatomas investigated in this study.

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CONTENTS

	<u>Page</u>
Chapter 1 Introduction -----	1
Chapter 2 Levels of Some Key Enzymes of DNA Synthesis in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell- lines -----	57
Summary -----	116
Chapter 3 Purification and Characterisation of Thymidine Kinases from Human Normal Liver and Hepatoma -----	118
Summary -----	188
Abbreviations -----	190
References -----	192

LIST OF FIGURES

Figure No.	Title	Page
1	Major Purine and Pyrimidine Deoxynucleotide Triphosphates -----	10
2	Metabolic Pathways for Biosynthesis and Degradation of Pyrimidine Nucleotides and the Biosynthesis of DNA -----	11
3	DNA Polymerase Activity (with Native DNA Template) in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	81
4	DNA Polymerase Activity (with Denatured DNA Template) in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	82
5	Ratio of DNA Polymerase Activities with Native and Denatured DNA Templates in Rat and Human Tissues, and Cell-lines -----	93
6	Thymidine Kinase Activity in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	84
7	Thymidylate Kinase Activity in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	85
8	Thymidylate Synthetase Activity in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	86
9	Deoxycytidylate Deaminase Activity in Rat and Human Normal Adult, Host, Cancerous and Foetal Tissues, and Cell-lines -----	87
10	Activities of Key Enzymes of DNA Synthesis in the Livers of Human Foetuses of Different Gestational Ages -----	94
11	Flow Diagram of Purification Stages used in the Partial Purification of Thymidine Kinases from Human Normal Liver and Hepatoma -----	131
12	The Elution Profile for Human Liver Thymidine Kinase on DE 52 Cellulose -----	133
13	The Elution Profile for Human Hepatoma Thymidine Kinase on DE 52 Cellulose -----	134
14	The Elution Profiles for Human Liver and Hepatoma Thymidine Kinases on Phosphocellulose P11 Columns -----	135
15	The Elution Profile for the Human Liver Thymidine Kinase II Preparation on Biogel A1.5 -----	137

Figure No.	Title	Page
16	The Elution Profile for the Human Hepatoma Thymidine Kinase II Preparation on Biogel A1.5 -----	138
17	The Elution Profiles for Human Liver and Hepatoma Thymidine Kinases on CM-Sephadex C50 Columns-----	139
18	The Elution Profiles for Human Liver and Hepatoma Thymidine Kinase IA Preparations on Biogel A1.5 -----	141
19	Calibration Curve for the Determination of the Molecular Weights of the Tdr Kinase Enzyme Forms from Human Normal and Cancerous Liver using a Biogel A1.5 column-----	150
20	pH Optimum Studies on Human Liver and Hepatoma Tdr Kinases -----	152
21	The Effect of Heating Normal Liver Tdr Kinase Enzyme Forms at 55°C for Various Time Intervals in the Presence and Absence of Thymidine -----	155
22	The Effect of Heating Hepatoma Tdr Kinase Enzyme Forms at 55°C for Various Time Intervals in the Presence and Absence of Thymidine-----	156
23	Ammonium Sulphate Inhibition of Tdr Kinases from Human Normal Liver and Hepatoma -----	157
24	Lineweaver-Burk Plots of Reciprocal Velocity Versus Reciprocal Tdr Concentration for Tdr Kinase IA from Human Normal Liver and Hepatoma -----	158
25	Lineweaver-Burk Plots of Reciprocal Velocity Versus Reciprocal Tdr Concentration for Tdr Kinase IB from Human Normal Liver and Hepatoma -----	159
26	Lineweaver-Burk Plots of Reciprocal Velocity Versus Reciprocal Tdr Concentration for Tdr Kinase IIA from Human Normal Liver and Hepatoma -----	160
27	Lineweaver-Burk Plots of Reciprocal Velocity Versus Reciprocal Tdr Concentration for Tdr Kinase IIB from Human Normal Liver and Hepatoma -----	161

LIST OF TABLES

Table No.	Title	Page
1	Summary of Purification and Stabilisation Studies on Thymidine Kinases -----	33
2	Summary of Studies on Physical Properties of Thymidine Kinases -----	38
3	Summary of Kinetic Studies on Thymidine Kinases -----	48
4	Deoxycytidylate Deaminase Activities of Rat and Human Normal and Cancerous Liver ---	78
5	A Comparison of the Levels of Key Enzymes of DNA Synthesis in Rat Normal and Host Livers and Transplantable 3'Me-DAB- induced Hepatomas -----	80
6	A Comparison of the Levels of Key Enzymes of DNA Synthesis in the NRH and PRH Rat Hepatoma Cell-lines -----	89
7	A Comparison of the Levels of Key Enzymes of DNA Synthesis in Human Adult Normal and Host Liver, Hepatoma, and Foetal Liver -----	91
8	A Comparison of the Levels of Key Enzymes of DNA Synthesis in AHH and PHH Human Hepatoma Cell-lines -----	95
9	A Comparison of the Levels of Key Enzymes of DNA Synthesis in Human Foetal, Host and Cancerous Oesophageal Tissue, and in a Human Oesophageal Cancer Cell-line (AHO) ---	97
10	A Comparison of the Levels of Key Enzymes of DNA Synthesis in Human Foetal Brain and Meningioma Tissue -----	99
11	Ratios of Activities of DNA Synthesis in Rat Hepatomas to those in Rat Normal Liver -----	105
12	Modifications to Reduce ATPase Activity in the Development of the Partial Purification Procedure for Thymidine Kinase from Human Normal and Cancerous Liver -----	127
13	Partial Purification of Human Normal Liver Thymidine Kinases -----	144
14	Partial Purification of Human Hepatoma Thymidine Kinases -----	145
15	The Relative Proportions of the Thymidine Kinase Forms in Human Normal Liver and Hepatoma -----	146

List of Tables (continued)

Table No.	Title	Page
16	Activities of Thymidylate Kinase, Thymidylate Phosphatase and Adenosine Triphosphatase Obtained in the Crude Fraction and at the Final Purification Stage of the Tdr Kinase Forms from Human Normal Liver -----	148
17	Activities of Thymidylate Kinase, Thymidylate Phosphatase and Adenosine Triphosphatase Obtained in the Crude Fraction and at the Final Purification Stages of the Tdr Kinase Forms from Human Hepatoma -----	149
18	pH Optima for Tdr Kinases in Human Normal Liver and Hepatoma -----	153
19	Summary of the Apparent K_m Values for Thymidine for Tdr Kinases from Human Normal and Cancerous Liver -----	162
20	Utilisation of Nucleoside and Deoxynucleoside Triphosphates as Phosphate Donors by Tdr Kinase Preparations from Human Normal and Cancerous Liver -----	164
21	Inhibition of Tdr Kinases from Human Normal and Cancerous Liver by Nucleoside and Deoxynucleoside Phosphates -----	165
22	The Ability of Thymidine Analogues to Serve as Substrates in the Tdr Kinase Catalysed Reaction -----	167
23	Effects of Thymidine Analogues on Human Liver and Hepatoma Tdr Kinases -----	168

CHAPTER 1

INTRODUCTION

	<u>Page</u>
<u>ENZYMES OF DNA SYNTHESIS IN NORMAL AND CANCEROUS LIVER</u> -----	3
<u>Human Primary Liver Cancer</u> -----	3
Nature, Incidence and Aetiology -----	3
Pathogenesis -----	5
Biochemical Changes -----	6
<u>Pathways of Pyrimidine Metabolism in DNA Synthesis</u> -----	9
Major <i>De Novo</i> and Salvage Pathways in Eukaryotes -----	9
The Role of Salvage versus <i>De Novo</i> Pathways in Pyrimidine Synthesis -----	12
DNA Synthesis in Foetal, Adult, Regenerating and Neoplastic Tissues, and Cells -----	13
Key Enzymes of DNA Synthesis in Mammalian Foetal, Adult, Regenerating and Tumorous Tissues, and Cell-lines -----	15
Ribonucleotide Reductase -----	15
Deoxycytidylate Deaminase -----	16
Thymidylate Synthetase -----	18
Thymidylate Kinase -----	20
DNA Polymerase -----	22
Thymidine Kinase -----	27
Control of the Key Enzymes of Pyrimidine and DNA Synthesis -----	31
<u>Purification and Properties of Thymidine Kinase</u> -----	32
Studies on the Purification and Stability of Thymidine Kinase -----	32
Purification -----	32
Stability -----	36
Physical Properties of Thymidine Kinase -----	37
Molecular Weight -----	40
Temperature Stability -----	41
pH Optimum -----	42
Divalent Metal Ion Requirements -----	43
Effects of Salts -----	44

	<u>Page</u>
Separation of Multiple Forms of Thymidine	
Kinase Activity -----	44
Electrophoresis -----	44
Ion-exchange Chromatography -----	46
Kinetic and Regulatory Properties of	
Thymidine Kinase -----	46
Apparent Michaelis Constants -----	47
Phosphate Acceptors -----	47
Phosphate Donors -----	50
Inhibitors -----	51
Regulation of Thymidine Kinase Activity -----	53
Aims of the Present Study-----	55

CHAPTER I

INTRODUCTION

ENZYMES OF DNA SYNTHESIS IN NORMAL AND CANCEROUS LIVER

HUMAN PRIMARY LIVER CANCER

Nature, Incidence and Aetiology

Primary liver cancer (hepatoma) is a carcinoma of the parenchymal cells of the liver. It is of particular interest in the area of human oncology since, unlike most other cancerous tissues, the quantities of normal and tumour material available from each patient in endemic areas are sufficient to allow detailed biochemical investigation. Studies on human hepatoma tissue have included the role of naturally occurring carcinogens, the relationship between liver tumours and embryonic liver tissues, and aspects of liver physiology and biochemistry related to the disease (Becker, 1974).

A very high incidence of primary liver cancer (98,2 per 100 000) is found amongst members of the Shangaan tribe from the east coast area of Mozambique, in East Africa (Kew, 1977). At the time of the present study, extensive use was made of migratory labour from this area by the gold mining industry in South Africa. Amongst these recruits, the incidence of hepatoma in Shangaan mine labourers out-numbered that of total Black recruits from South Africa by 6,6:1 (Geddes and Falkson, 1970), although the Shangaan group constituted only about 50% of the Black mine-labourers.

The mean age at which Southern African Black patients present clinically with hepatomas is about 33 years, whereas in non-endemic areas primary liver cancer has been found to occur most commonly in the fifth to seventh decades (Geddes and Falkson, 1970; Kew, 1977). Men are more often affected than women, the ratio for African Black patients being

5 or 6:1 (Kew, 1977). The onset of the disease in Black patients is very rapid with a history of symptoms of only 4 to 6 weeks. The disease is usually at an advanced stage at the time of presentation and the prognosis is extremely grave, mortality being almost 100%, with survival times of only 4 to 6 months from the onset of symptoms (Geddes and Falkson, 1970; Kew, 1977). Because of metastases and gross liver involvement by these carcinomas, surgical intervention is seldom contemplated (Terblanche, 1977). Disappointing results have been obtained during chemotherapeutic trials (Falkson, 1975; World Health Organisation, 1977).

It appears that hepatocellular carcinoma occurs in two forms (Kew, 1977). In most parts of the world the tumour is rare, predominantly occurs in elderly people, and is generally associated with chronic cirrhosis of the liver. On the other hand the tumour is common amongst the indigenous peoples of Sub-Saharan Africa and the Far East, occurs primarily in young adults, and is characterised by acute presentation and a rapid course. The latter tumour is not always associated with cirrhosis. There is no known genetic predisposition to hepatoma and it has been suggested that the aetiology of the two types of tumour is different.

Analysis of the possible aetiological factors responsible for the exceptionally high incidence of hepatoma amongst the Blacks of East Africa has implicated the contamination of ground nuts by aflatoxins which are produced by the fungus *Aspergillus flavus* (Oettlé, 1964; Brit. med.J.Editorial, 1975; Van Rensburg *et al.*, 1974). Ground nuts form a staple part of the diet in these areas and there is evidence that the levels of aflatoxins ingested are within the estimated carcinogenic range for humans (Alpert *et al.*, 1971; Becker, 1974). It has also been demonstrated that events related to hepatitis B viral infection, including the presence of the hepatitis B surface antigen (HBs antigen) and antibody

against the hepatitis B core antigen (anti-HBc), show a higher frequency in hepatoma patients than in matched controls (Kew *et al.*, 1977; Larouzé *et al.*, 1977). It remains to be shown that the persistence of the hepatitis B virus in patients with hepatomas is not merely attributable to the presence of the tumour, and that both the viral infection and the tumour are not manifestations of an underlying disturbance in the immune mechanism. The modes of action of these possible causative or predisposing factors in the development of hepatomas have not yet been elucidated. It is, however, possible that an oncogenic virus and aflatoxin may act together in the production of primary liver cancer. Repeated exposure to a dietary carcinogen in patients with low grade hepatitis, due to hepatitis B viral infection, may conceivably result in the development of a hepatoma. It has been shown by Lin *et al.* (1974) that marmosets with a low grade hepatitis are more prone to develop aflatoxin-induced hepatomas.

Of further interest is the finding of a possible permissive or promotive role for male hormones. Johnson *et al.* (1972) have described the spontaneous development of hepatomas in patients with aplastic anaemia on treatment with androgenic-anabolic steroids. Similarly, development of spontaneous hepatomas in mice is higher in males than in females (Johnson *et al.*, 1972). The incidence of spontaneous development of hepatomas in male mice is decreased following orchidectomy (Andervont, 1952). These findings correlate with the higher incidence of hepatomas in Black males in Southern Africa.

Pathogenesis

Liver cells are susceptible to the effects of a number of diverse carcinogens and also to ionising radiation (Farber, 1974a & b). Many of the hepatocarcinogens require metabolic conversion to more

reactive forms, which are then capable of binding to various cell constituents such as DNA, RNA, or protein. Generally it appears that chemical carcinogens do not induce hepatomas directly, but rather that they trigger off a chain reaction leading ultimately to a malignant neoplastic condition. Farber (1974a) has postulated that this process involves a rapid 'initiation' step followed by a more prolonged period of neoplastic development, termed 'promotion', during which a series of cell populations progressively evolves towards a malignant condition. The process of initiation probably involves damage to cellular DNA by the carcinogen. This damage would normally be subject to repair. However, if the affected cells are simultaneously in a state of proliferation or regeneration, as might occur during a viral hepatitis infection, damage to cellular DNA may be rendered permanent by replication before repair can be effected. The carcinogens themselves may also exert a direct inhibitory effect on the enzymes of DNA repair. It has been shown that treatment of human fibroblasts with Aflatoxin B produces DNA lesions and appears to inhibit excision repair of DNA (Sarasin *et al.*, 1977). The chemically modified cells might be rendered non-viable or might produce new cell populations with biochemical properties which allow neoplastic growth. Selective growth of these new cells could be enhanced if they are capable of proliferation in the presence of the carcinogen, while the division of normal hepatocytes is inhibited. Farber (1974a) has suggested that these new proliferating cells might form isolated 'hyperplastic' nodules, which could then serve as precursors for the final series of stages towards the development of a malignant hepatoma.

Biochemical Changes

Biochemical approaches directed towards chemotherapy of neoplastic diseases are essentially concerned with the late or terminal stages of

cancer progression. However, the ability to recognise the earlier biochemical lesions associated with neoplasia would allow a shift in the emphasis in chemotherapy towards an approach directed at the initial stages of carcinogenesis.

The development of a rat transplantable hepatoma which shows normal or near normal levels of the enzymes characteristic of differentiated liver parenchymal cells (Morris, 1965), together with a spectrum of solid tumours of this type which shows different degrees of malignancy, have provided useful models for the study of biochemical differences between normal and cancerous liver tissues. These tumours were induced by feeding low doses of hepatocarcinogens to inbred strains of rats. On the assumption that the rate of cellular proliferation is indicative of the degree of malignancy, and that tumour growth rate is a readily measurable parameter of malignancy, Weber (1971 and 1972) has classified the Morris series of rat transplantable hepatomas into three broad groups, namely: hepatomas with slow growth rates, which are capable of producing host death within 3 to 12 months; those which show intermediate growth rates and are capable of causing host death within 4 to 6 weeks; and the rapidly growing hepatomas which cause death of the host rat within 1 to 3 weeks. In addition three types of relationship between biochemical changes and the degree of malignancy shown in the Morris rat hepatomas have been noted (Weber, 1977a and 1977b). Firstly there are those biochemical parameters which exhibit changes paralleling the degree of malignancy of each tumour, these being termed 'progression-linked' alterations. Secondly, there are 'transformation-linked' alterations in which biochemical parameters are altered to a similar degree, irrespective of the malignancy of the tumour. Thirdly, there are

'coincidental changes' in biochemical parameters which bear no relation to the degree of malignancy.

In rat hepatomas the key regulatory enzymes of glycolysis (ie. those subject to precise regulation and control) show increased activity with increasing tumour growth rate, whereas the activity of the key gluconeogenic enzymes decreases, indicating a progressive predominance of glycolysis over the pathways of gluconeogenesis in carbohydrate metabolism (Weber, 1974). Fundamentally similar results have been reported for enzymes of glycolysis and gluconeogenesis (Balinsky *et al.*, 1973a,b,c) and for some key enzymes of DNA, RNA, purine, pyrimidine, protein, and urea synthesis in human hepatomas (Weber *et al.*, 1976b).

In his Molecular Correlation Concept, Weber (1966, and 1977a and b) has suggested that the specific pattern of biochemical changes occurring in rapidly-dividing cells can be related to ordered alterations in the regulation of the cell-genome. Altered regulation at the metabolic level is effected by inverse changes in the activity of groups of key enzymes of the synthetic and catabolic pathways. The Molecular Correlation Concept proposes that the synthesis of these groups of key enzymes is controlled by functional gene units, and that the control of expression of these gene units directly regulates the metabolic state of a given cell. In hepatoma cells the changes in the balance of metabolic pathways are thus ascribed to an altered expression of these functional gene units.

Potter (1965) has proposed a theory of oncogeny as blocked ontogeny, which suggests that rat hepatoma cells represent parenchymal cells which have been blocked at various stages of differentiation. Thus the minimal deviation rat hepatomas would represent a state of differentiation very close to that found in differentiated adult rat liver, whereas the rapidly-

growing hepatomas would more closely resemble undifferentiated parenchymal stem-cells.

PATHWAYS OF PYRIMIDINE METABOLISM IN DNA SYNTHESIS

The biosynthesis of DNA (replication) requires the presence of four deoxynucleoside-triphosphates, each consisting of a purine (adenine or guanine) or pyrimidine (cytosine or thymine) base, attached to a deoxyribose-triphosphate moiety (Figure 1).

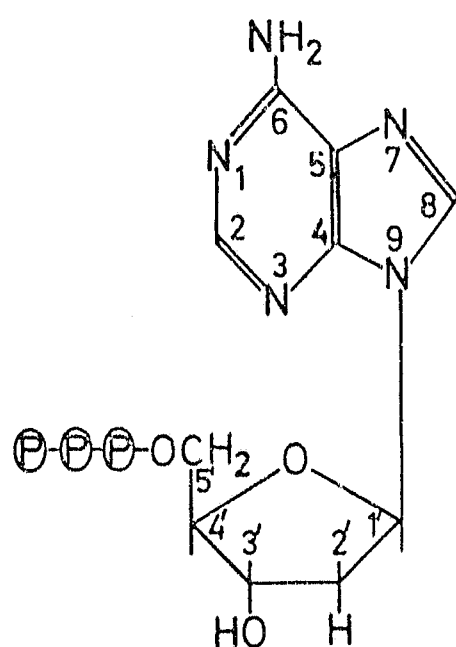
Major *De Novo* and Salvage Pathways in Eukaryotes

Thymidine (5-methyl-uridine) is specific for DNA synthesis, being replaced by uridine in RNA. Studies on the metabolism of this base are therefore of particular relevance in studies concerned with DNA synthesis. Since DNA synthesis is template directed, a shortage or absence of a single base may adversely affect replication. The deoxyribonucleotide precursors of DNA synthesis are normally present in very low concentrations, being synthesised as required with very little accumulation. These precursors are probably synthesised during the intermitotic phase (S phase) of the cell cycle for direct incorporation into DNA.

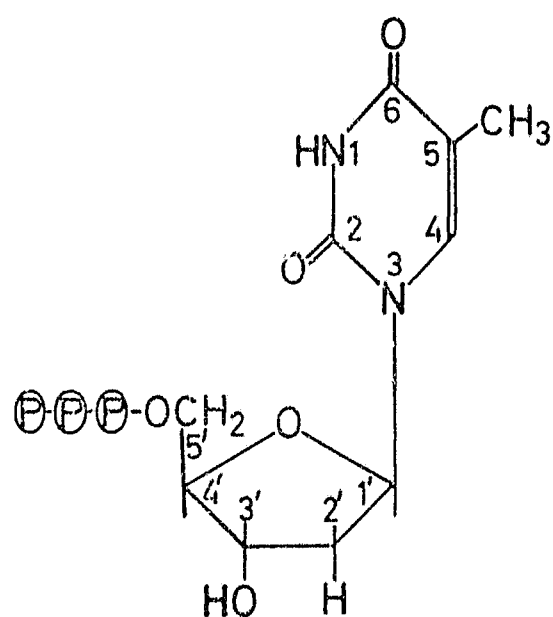
The pyrimidine deoxyribonucleotides dCTP and dTTP are primarily derived from the ribonucleotide UTP (Henderson and Paterson, 1973, p.217). From Figure 2 it can be seen that UMP is synthesised from aspartate and carbamylphosphate, which are converted to ureido-succinate, dihydro-orotate, orotate, ornithine-monophosphate and finally uridine-monophosphate, by the enzymes aspartate transcarbamylase, dihydro-orotase, dihydro-orotate dehydrogenase, orotate pyrophosphorylase, and orotidylate decarboxylase, respectively. UMP may also be synthesised via a salvage pathway, by the conversion of uridine to UMP in the presence of the enzyme uridine kinase which catalyses the phosphorylation of uridine. The opposing catabolic pathways allow UMP to be degraded to β -alanine,

FIGURE 1

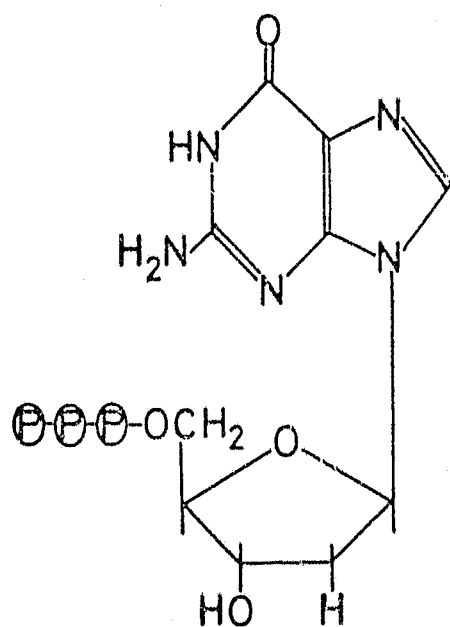
MAJOR PURINE AND PYRIMIDINE DEOXYNUCLEOTIDE TRIPHOSPHATES



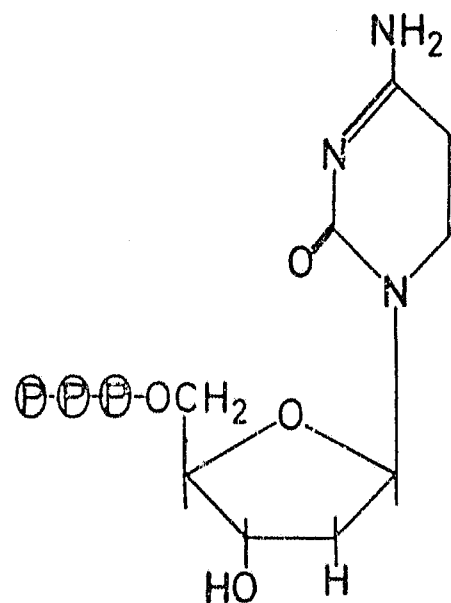
2'-DEOXYADENOSINE 5'-TRIPHOSPHATE



2'-DEOXYTHYMIDINE 5'-TRIPHOSPHATE



2'-DEOXYGUANOSINE 5'-TRIPHOSPHATE



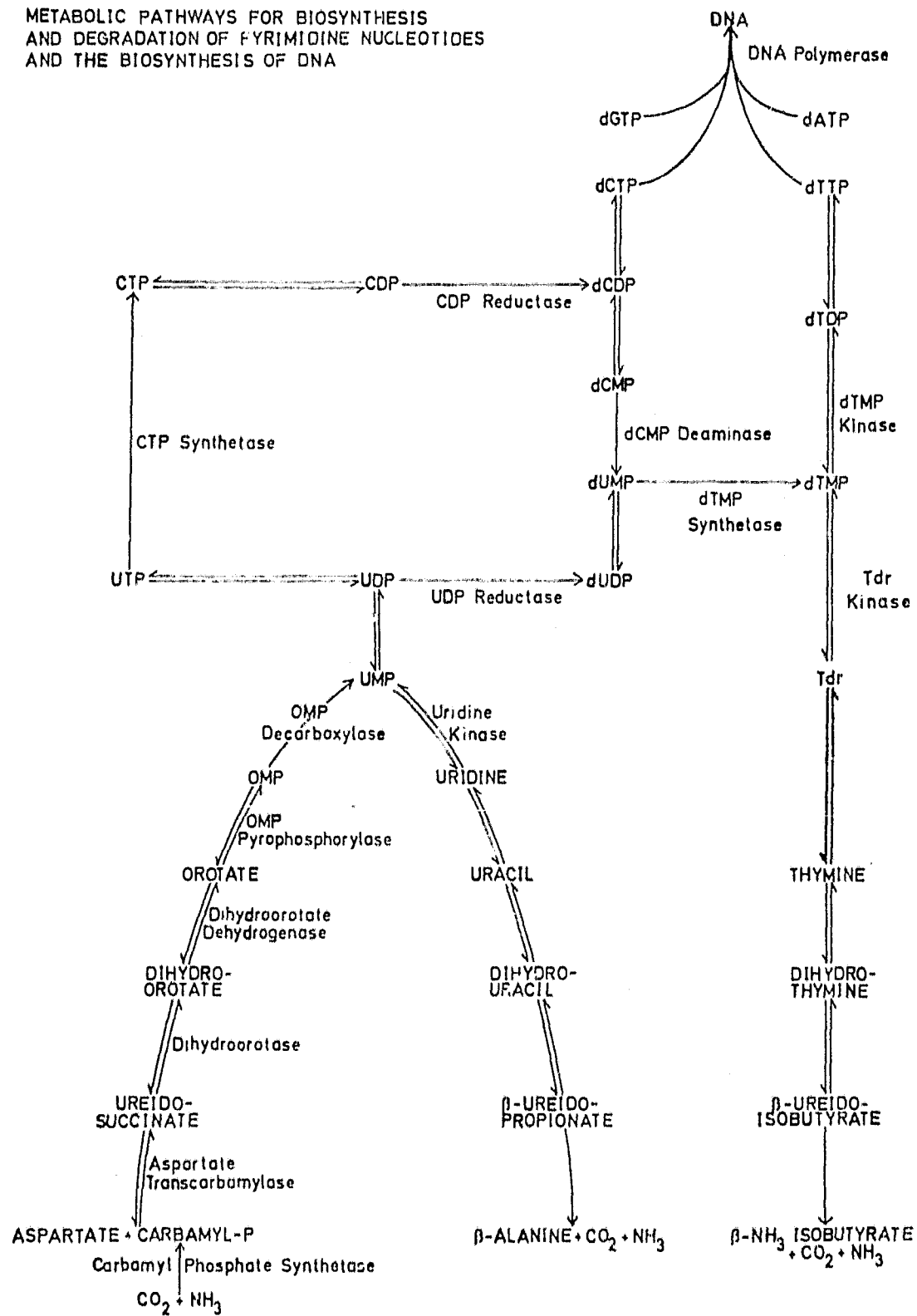
2'-DEOXYCYTIDINE 5'-TRIPHOSPHATE

The numbering systems for purines and pyrimidines are indicated for respectively dATP and dTTP.

FIGURE 2.

11.

METABOLIC PATHWAYS FOR BIOSYNTHESIS
AND DEGRADATION OF PYRIMIDINE NUCLEOTIDES
AND THE BIOSYNTHESIS OF DNA



CO₂ and ammonia. UMP for deoxynucleoside-triphosphate synthesis is phosphorylated by the enzyme UMP kinase to form UDP. This UDP may either be reduced to dUDP in the presence of ribonucleotide reductase or it may be further phosphorylated to UTP and then converted to CTP via the enzyme CTP synthetase. CTP may be dephosphorylated in the presence of CTPase to form CDP, which in turn may be reduced to dCDP, again via the enzyme ribonucleotide reductase. dCDP may either be phosphorylated to form dCTP or dephosphorylated to dCMP, which can be deaminated in the presence of the enzyme deoxycytidylate deaminase (dCMP deaminase) to form dUMP. Although dCMP deaminase has been assigned a role in the major pathway of dTTP synthesis (Maley and Maley, 1960; Bresnick, 1974), it has been suggested by Sneider and Potter (1969) that this enzyme may also have a salvage role, in that 5-methyl-dCMP (from DNA catabolism) may be deaminated in the presence of dCMP deaminase, leading to direct production of dTMP.

The *de novo* pathway of dTMP synthesis involves the conversion of dUMP to dTMP in the presence of the enzyme thymidylate synthetase (dTMP synthetase). The dTMP may be further phosphorylated in the presence of thymidylate kinase (dTMP kinase) to form dTDP, and this may finally be converted to dTTP in a reaction catalysed by a non-specific nucleoside diphosphokinase. dTMP may, in addition, be synthesised via a salvage pathway in which Tdr derived exogenously or from dTMP catabolism is phosphorylated in the presence of the enzyme thymidine kinase (Tdr kinase).

The final incorporation of the nucleoside-triphosphate bases into DNA is catalysed by the enzyme deoxyribonucleic acid polymerase (DNA polymerase).

The Role of Salvage versus *De Novo* Pathways in Pyrimidine Synthesis

It has been noted by Roux (1973) that the major pathway of dTTP synthesis is generally similar in all normal cells, whereas the salvage

CO₂ and ammonia. UMP for deoxynucleoside-triphosphate synthesis is phosphorylated by the enzyme UMP kinase to form UDP. This UDP may either be reduced to dUDP in the presence of ribonucleotide reductase or it may be further phosphorylated to UTP and then converted to CTP via the enzyme CTP synthetase. CTP may be dephosphorylated in the presence of CTPase to form CDP, which in turn may be reduced to dCDP, again via the enzyme ribonucleotide reductase. dCDP may either be phosphorylated to form dCTP or dephosphorylated to dCMP, which can be deaminated in the presence of the enzyme deoxycytidylate deaminase (dCMP deaminase) to form dUMP. Although dCMP deaminase has been assigned a role in the major pathway of dTTP synthesis (Maley and Maley, 1960; Bresnick, 1974), it has been suggested by Sneider and Potter (1969) that this enzyme may also have a salvage role, in that 5-methyl-dCMP (from DNA catabolism) may be deaminated in the presence of dCMP deaminase, leading to direct production of dTMP.

The *de novo* pathway of dTMP synthesis involves the conversion of dUMP to dTMP in the presence of the enzyme thymidylate synthetase (dTMP synthetase). The dTMP may be further phosphorylated in the presence of thymidylate kinase (dTMP kinase) to form dTDP, and this may finally be converted to dTTP in a reaction catalysed by a non-specific nucleoside diphosphokinase. dTMP may, in addition, be synthesised via a salvage pathway in which Tdr derived exogenously or from dTMP catabolism is phosphorylated in the presence of the enzyme thymidine kinase (Tdr kinase).

The final incorporation of the nucleoside-triphosphate bases into DNA is catalysed by the enzyme deoxyribonucleic acid polymerase (DNA polymerase).

The Role of Salvage versus *De Novo* Pathways in Pyrimidine Synthesis

It has been noted by Roux (1973) that the major pathway of dTTP synthesis is generally similar in all normal cells, whereas the salvage

pathways show greater diversity in both their nature and distribution. The relative contributions of the *de novo* or major pathway and the salvage pathways depend on the organism and on the requirements of the cells concerned at a given time (Sneider and Potter, 1969). It has been noted by these authors and by Goodman and Potter (1973) that the effectiveness of a chemotherapeutic agent may depend upon the existence of alternative metabolic pathways (whether *de novo* or salvage) in corresponding normal and cancerous tissues. They concluded that the ultimate fate of a given metabolite varies both in different tissues, and within the same tissue under varying conditions. For example, blocking the major pathway to dTTP in human lymphoblasts by inhibiting thymidylate synthetase activity with the antifolate drug methotrexate leads to a 3-fold elevation in Tdr kinase activity (Chello *et al.*, 1976).

Although Tdr kinase has been assigned a salvage role in DNA synthesis, Weber (1977b) has suggested that this enzyme is a key enzyme of DNA synthesis because of the importance of dTMP in DNA anabolism.

DNA Synthesis in Foetal, Adult, Regenerating and Neoplastic Tissues, and Cells

Rat post-natal developing liver exhibits high levels of DNA synthesis which decrease, within about 10 weeks, to the levels found in adult rat liver (Köhler, 1972). Following partial hepatectomy adult rat liver can regain 80% of its weight, protein and DNA within 4 days. During this period the incorporation of labelled Tdr into liver tissue has been shown to increase by some 23-fold relative to normal rat liver (Hopkins *et al.*, 1973).

The opposing pathways of thymidine utilisation in differentiated, regenerating and neoplastic rat liver have been compared by simultaneously measuring the incorporation of labelled thymidine into DNA and the degra-

dation of thymidine to carbon dioxide (Ferdinandus *et al.*, 1971).

Liver tissue from neonatal rats shows a very high incorporation of Tdr into DNA as well as high activity for the pathway of thymidine catabolism. As the adult stage of liver development is approached, the anabolic pathways of thymidine are decreased and catabolism of thymidine is increased. Partial hepatectomy produces an increase in the incorporation of thymidine into DNA with a simultaneous decrease in thymidine breakdown. Both metabolic changes last for about 96 hours before returning to levels resembling those found in normal adult rat liver. In the Morris spectrum of transplantable rat hepatomas the incorporation of Tdr into DNA and the degradation of Tdr to CO_2 have been found to be, respectively, directly and inversely proportional to the tumour growth rate.

The metabolic imbalance in the pathways of DNA synthesis and deoxynucleotide catabolism suggests a pattern in these malignancy-linked phenotypic expressions of neoplasia, in that it appears that enzymes having the lowest activities in resting, differentiated liver show the greatest increase in rapidly growing hepatoma tissue. Conversely, those enzymes with high levels of activity in normal adult tissue show little or no alteration in the corresponding neoplastic tissue (Weber, 1974; Weber *et al.*, 1976a). Phenotypic evidence for the reprogramming of gene expression in nucleic acid metabolism is provided by increases in the key enzymes of UMP, dTTP and DNA synthesis, and decreases in the enzymes of UMP and Tdr catabolism, both of which parallel increasing tumour growth rate.

Studies using human tissues have revealed similar correlations between growth rate, or developmental stage, and DNA synthesis. Patterns of DNA and RNA synthesis in human foetal liver, kidney, heart, spleen,

lung and brain, obtained between 8 to 15 weeks gestation, have been investigated by measuring the incorporation of ^3H -Tdr into DNA and ^3H -Udr into RNA (Mukherjee and Hastings, 1973). Each of the different organs appears to show a unique periodicity of nucleic acid synthesis over the period 8 to 15 weeks gestation, with an inverse relationship between DNA and RNA synthesis.

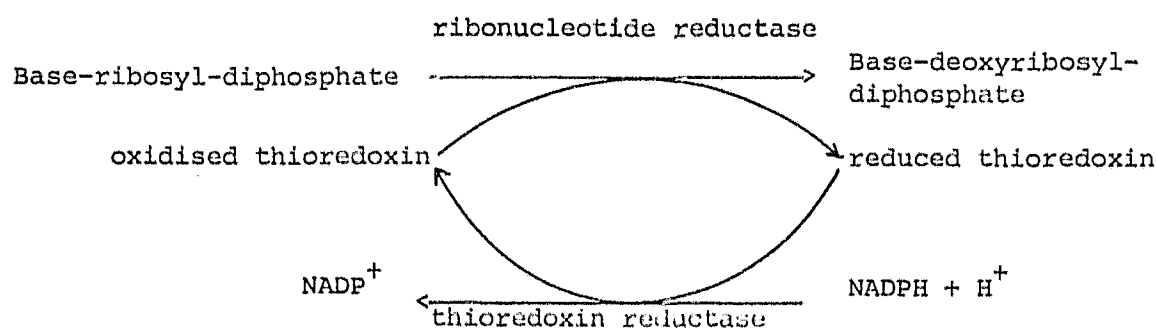
Studies of DNA synthesis have recently been extended to human hepatomas. Using samples of biopsied human hepatoma, the patients being two males (aged 43 and 62) and two females (aged 22 and 25), it has been shown that the tumour tissues failed to exhibit an increase in the net incorporation of Tdr into DNA, but that there was a small decrease in Tdr catabolism (Weber et al., 1976b). On the basis of these findings and of studies on the key enzymes of glycolysis, gluconeogenesis, purine synthesis and the urea cycle, these authors concluded that human primary hepatomas show a low to medium growth rate and malignancy.

Key Enzymes of DNA Synthesis in Mammalian Foetal, Adult, Regenerating and Tumorous Tissues and Cell-lines

Pyrimidine and DNA metabolism are closely correlated with cellular replication and growth rate. Studies have been undertaken to determine the relationship between key enzymes of pyrimidine and DNA synthesis and the degree of neoplastic transformation.

Ribonucleotide Reductase (2'-deoxyribonucleoside-diphosphate:oxidised-thioredoxin 2'-oxidoreductase, EC 1.17.4.1)

Ribonucleotide reductase is an allosteric, rate-limiting enzyme which catalyses the reduction of ribosyl groups of the nucleoside diphosphates CDP, UDP, ADP and GDP, in the presence of reduced thioredoxin, as shown on page 16.



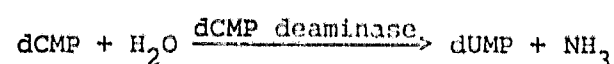
Reduced thioredoxin is regenerated by the enzyme thioredoxin reductase in an NADPH-requiring reaction.

Rat embryonic and hepatoma ribonucleotide reductases are activated by dTTP and inhibited by excess dATP (Moore and Hurlbert, 1966; Murphree *et al.*, 1968). These nucleotides are believed to regulate enzyme activity allosterically. Specificity for substrates (ADP, GDP, CDP, UDP) is also determined by nucleoside triphosphate allosteric effectors (Brown and Reichard, 1969). The enzyme from mouse L-cells has been shown to have a half-life of 2 hours (Turner *et al.*, 1968).

The levels of ribonucleotide reductase activity are very low in rat normal liver as well as in the host liver of rats bearing transplantable rat hepatomas. However the activity of this enzyme is very high in foetal rat liver and in the Morris series of transplantable hepatomas. In the latter case the enzyme activity shows a very close correlation with the growth rate of the tumours (Elford *et al.*, 1970).

Deoxycytidylate Deaminase (dCMP aminohydrolase, EC 3.5.4.12)

dCMP deaminase catalyses the deamination of dCMP to form dUMP (Maley, F. and Maley, G.F., 1961), as shown below:



dCMP deaminase uses as substrates dCMP, 5-methyl-dCMP, 5-hydroxymethyl-dCMP, and the 5-halo derivatives of dCMP. The enzyme is subject to allosteric regulation by dCTP and dUMP (Henderson and Paterson, 1973, p.236), dCTP serving as an activator, and dTTP as an inhibitor.

dCMP deaminase has been demonstrated in a number of animal tissues and cells such as chick embryo, and monkey and rabbit liver (Maley, 1967); rat liver and hepatomas (Sneider *et al.*, 1969); hamster kidney cells (Rolton and Keir, 1974a); in a variety of human normal and neoplastic tissues (Giusti *et al.*, 1970); and in human Chang liver cells (Eker, 1968).

In adult rat tissues the enzyme is present in rate-limiting amounts (Bresnick, 1974). The levels of the enzyme have been shown to increase during periods of increased cell division (Maley, F. and Maley, G.F, 1961). Ferdinandus *et al.* (1971) have described very high levels of dCMP deaminase in neonatal rat liver, these being some 28-fold higher than in adult liver. This activity decreases rapidly within 2 weeks after birth and reaches adult levels within 8 weeks. The decrease is considered to represent a repression of the genes responsible for the synthesis of dCMP deaminase. dCMP deaminase activity increases in rat liver following partial hepatectomy (Maley *et al.*, 1965). If this post-hepatectomy increase is blocked with Actinomycin D, the subsequent onset of DNA synthesis and cell division is also delayed. There is a correlation between enzyme activity and tumour growth rate in the Morris series of rat transplantable hepatomas (Sneider *et al.*, 1969; Ferdinandus *et al.*, 1971). The more slowly growing tumours have levels of dCMP deaminase activity similar to those of adult normal liver, whereas very high levels of activity are present in rapidly growing Novikoff rat hepatomas.

It has been suggested that the dCMP deaminase pathway might be implicated in an alternative route to dTMP synthesis, involving the deamination of 5-methyl-dCMP, that is, a salvage route which by-passes the conversion of dUMP to dTMP catalysed by thymidylate synthetase

(Sneider and Potter, 1969). In this respect it has been shown that up to 75% of dTTP in *E. coli* cells may be produced via the deamination of 5-methyl-dCMP (Karlström and Larsson, 1967). Maley, G.F. and Maley, F. (1961) have shown that ^{32}P -dCMP is incorporated into both cytidine and thymidine of DNA in chick embryo extracts.

Infection of baby hamster kidney cells with Herpes simplex virus leads to the appearance of a new dCMP deaminase which shows properties different from those of the host cell enzyme (Rolton and Keir 1974a). This may represent either the induction of a new protein which is encoded in the viral DNA, or an infection-produced derepression of a host cell form of the enzyme which is not normally expressed.

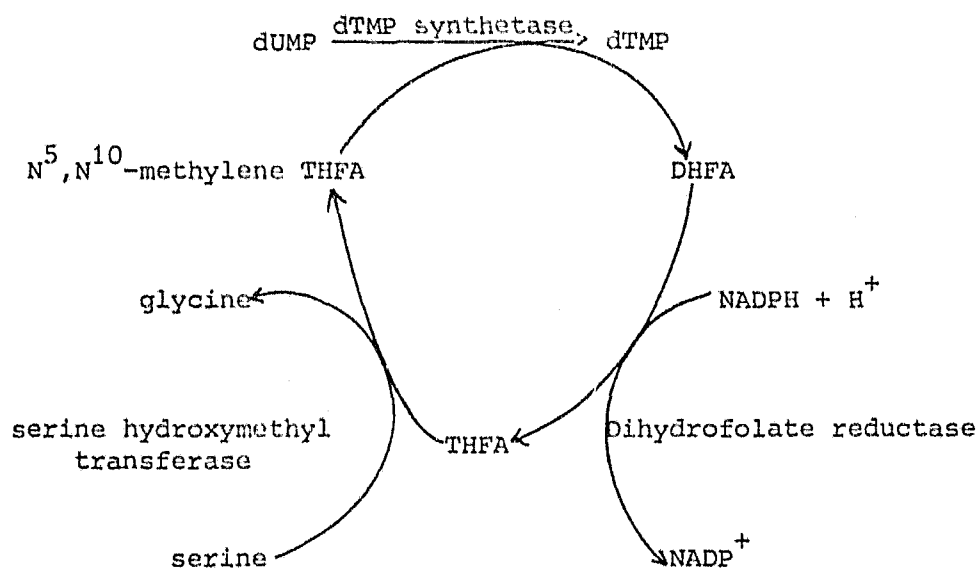
Levels of dCMP deaminase activity have been measured in human normal and neoplastic tissues (Giusti *et al.*, 1970). These were low in normal human tissues with the exception of liver, which showed high dCMP deaminase activity. In benign tumours levels of dCMP deaminase activity were similar to those in the corresponding normal tissues, whereas malignant tumours such as melanomas; carcinomas of the skin, breast, lung and uterus; adenocarcinomas of the ovary, colon and rectum; brain tumours; and a malignant neuroma, exhibited very high dCMP deaminase activities.

Serum dCMP deaminase levels have been found to be raised in all acute pathological conditions of the liver, during the course of acute infections, and following surgery (Miller and Ressler, 1969).

Thymidylate Synthetase ($\text{N}^5, \text{N}^{10}$ -methylene-tetrahydrofolate:2'-deoxy-uridylate C-methyltransferase, EC 2.1.1.-.).

dTMP synthetase plays an important role in DNA synthesis, since it catalyses the conversion of dUMP to dTMP which is the first committed step in the *de novo* pathway leading to dTTP. The conversion of dUMP to

dTMP requires the presence of the cofactor N^5,N^{10} -methylene-tetrahydrofolic acid, which donates a methylene group to the 5-carbon position on the uracil ring. The methylene group is then reduced to form a methyl group. Dihydrofolic acid (DHFA) formed in this reaction is subsequently reduced to tetrahydrofolic acid (THFA) in the presence of NADPH and the enzyme dihydrofolate reductase. THFA is converted to N^5,N^{10} -methylene-THFA by the serine-requiring enzyme serine hydroxymethyl transferase as shown below:



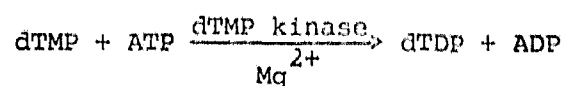
Although mono-, di-, and triphosphate derivatives of adenosine, cytidine, guanosine, and uridine, and the corresponding deoxynucleoside forms have been reported to have no effect on dTMP synthetase activity from chick embryos (Lorenson *et al.*, 1967), the enzyme from cultured Chinese hamster cells appears to be weakly inhibited by dTMP (Conrad and Ruddle, 1972). dTMP synthetase activity is labile. Increased dTMP synthetase activity detectable during periods of DNA synthesis and cell division may be blocked by Actinomycin D or puromycin (Conrad and Ruddle, 1972), suggesting that increased activity of the enzyme is dependent upon transcription of genetic information and subsequent protein synthesis.

The anti-cancer drug 5-fluoro-uracil inhibits dTMP synthetase via its metabolic derivative 5-fluoro-deoxyuridylate (5-F-dUMP), which is an analogue of the normal substrate dUMP. In addition the anti-cancer drug methotrexate inhibits dihydrofolate reductase and therefore blocks dTMP synthetase activity by preventing the regeneration of tetrahydrofolic acid.

dTMP synthetase activity is very low or undetectable in adult normal rat liver and other non-proliferating tissues (Hartmann and Heidelberger, 1961). Levels of dTMP synthetase activity have been shown to be increased in minimal deviation rat transplantable hepatomas, and there is a very good correlation between enzyme activity and tumour growth rate in the Morris series of rat transplantable hepatomas (Sneider *et al.*, 1969). In rapidly growing Morris hepatomas dTMP synthetase activity is elevated some 15-fold compared with host rat liver, whereas the activity of the enzyme in Novikoff hepatoma tissue is 25-fold higher than in rat normal liver (Weber, 1974). It has been calculated that levels of dTMP synthetase increase approximately 5-fold in 24-hour rat regenerating liver (Weber, 1974). Because of the low levels of dTMP synthetase, even in dividing cells, it has been suggested that this enzyme is rate-limiting in the pathway of dTTP synthesis (Weber, 1976a).

Thymidylate Kinase (ATP:deoxythymidine-monophosphate
phosphotransferase, EC 2.7.4.9).

dTMP kinase catalyses the phosphorylation of dTMP to form dTDP in the presence of ATP and Mg^{2+} as shown below:



Due to the presence of a very active nucleoside diphosphate kinase, dTDP is rapidly converted to dTTP. This led initially to the

idea that dTMP might be pyrophosphorylated to form dTTP directly.

However this was disproved by the finding that isotopic label was not incorporated into dTTP when β -³²P-ATP was used as a phosphate donor for Novikoff rat hepatoma dTMP kinase (Grav and Smellie, 1963; Ives, 1965). dTMP kinase from Chang human liver cells has been shown to be subject to inhibition by dTTP and dTDP (Eker, 1968), although it does not appear to be allosterically regulated (Kielley, 1970).

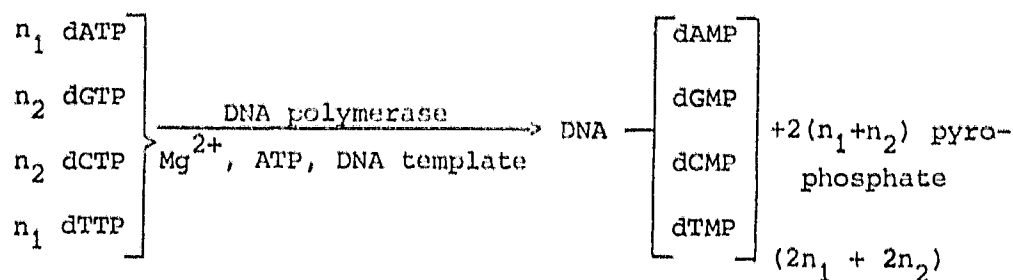
The activity of dTMP kinase in cultured Chang human liver cells appears to correlate with the cellular growth phase, being highest during periods of rapid cell proliferation (Eker, 1968). In addition the induction of DNA replication in human lymphocytes by phytohaemagglutinin stimulation is accompanied by increased dTMP kinase activity (Loeb *et al.*, 1970). The levels of dTMP kinase activity in non-dividing rat liver are low (Nakamura and Sugino, 1966). Levels of enzyme activity in neonatal rat liver have been found to be high up to the fifth day after birth, with a subsequent decline to low adult levels within about 8 weeks (Sneider and Potter, 1968). Ferdinandus *et al.* (1971) have observed that these results indicate that levels of activity of dTMP kinase in neonatal rat liver are about 131-fold higher than in adult rat liver. Enzyme activity is increased 16-fold in 24-hour rat regenerating liver when compared with normal rat liver, and is 71-fold greater in Novikoff rat hepatoma tissue than in adult rat liver (Weber, 1974). Using rat transplantable hepatomas of different growth rates, Bukovsky and Roth (1963) reported that dTMP kinase activity did not appear to correlate with growth rate. On the other hand Sneider *et al.* (1969) have assayed dTMP kinase activity in the Morris spectrum of transplantable rat hepatomas and have observed some degree of correlation with malignancy. Rats bearing implanted Yoshida sarcomas or AH 130 tumours

showed increased levels of dTMP kinase in both host liver and spleen (Shirasaka and Fujii, 1975). Since there was no increase in serum dTMP kinase levels these authors suggested that the observed changes in liver and splenic tissues represented a *de novo* synthesis of the enzyme in these host tissues, in response to substances released by the tumour tissues.

dTMP kinase activity has been shown to be increased, in comparison with host tissues, in the following human tumours: glandular carcinomas of the caecum, rectum and breast; squamous cell carcinomas of the tonsil, tongue, larynx and lung; uterine adenocarcinoma; sarcomas; and kidney hypernephromas, (Gordon *et al.*, 1968). Although limited sample numbers of each tissue were assayed and relatively low increases in enzyme activity were measured (1.2 to 8.0-fold), the activity of dTMP kinase appeared to be consistently increased in all of these tumours when compared with appropriate normal tissues.

DNA Polymerase (Deoxynucleoside-triphosphate:DNA deoxynucleotidyl-transferase, EC 2.7.7.7).

DNA Polymerase catalyses the template-directed incorporation of purine and pyrimidine deoxyribonucleoside triphosphates into DNA. The reaction requires the presence of Mg^{2+} , ATP, an adequate supply of each of the four deoxynucleoside triphosphates dATP, dGTP, dCTP, dTTP, and suitable DNA template, as shown below:



DNA polymerase from rat regenerating liver has been found to have a pH optimum of 8.0 and to be capable of using both native or heat-

denatured DNA as a template (Mantsavinos, 1964). The enzyme isolated from rat liver and transplantable hepatomas exists in multiple molecular forms with different subcellular localisation, molecular weights, and template DNA requirements. The higher molecular weight form requires denatured template DNA while the lower molecular weight form is more active with native template DNA (Baril and Laszlo, 1970).

Considerable confusion exists in the literature concerning the nomenclature of the various forms of DNA polymerase as defined by their subcellular localisation, physical properties, and template DNA requirements. Weissbach *et al.* (1975), Weissbach (1977) and Wintersberger (1977) have proposed the following nomenclature for the major DNA-replicating forms of eukaryotic DNA polymerase:

DNA polymerase α , the major fraction of DNA polymerase activity, is found largely, if not entirely, in the cytoplasm. During DNA synthesis high concentrations of this enzyme are associated with the nuclear fraction, and it has been suggested that the cytoplasmic localisation of DNA polymerase represents an artifact due to cell-fractionation. Alternatively it is possible that α -polymerase activity is located in the cytoplasm of non-replicating cells and is actively transported from the cytoplasm to the nucleus at the onset of DNA replication. The active enzyme from human KB-cells has a molecular weight of about 170 000 and appears to exist as a dimer, each monomer having a molecular weight of about 87 000 (Sedwick *et al.*, 1975). DNA polymerase α requires sulphhydryl groups for activity and is strongly inhibited by N-ethylmaleimide and p-chloromercuribenzoate. This enzyme form shows increased activity in dividing cells and is inhibited by cytosine arabinoside triphosphate. DNA polymerase α requires a polydeoxynucleotide chain as a template and a 3'-hydroxyl group of an oligodeoxynucleotide or an oligoribonucleotide chain as a primer.

This enzyme form appears to correspond to the high molecular weight DNA polymerase described by Baril and Laszlo (1970).

DNA polymerase β is a low molecular weight form of the enzyme which is found primarily in the nuclear fraction. The enzyme consists of a single polypeptide chain with a molecular weight of about 45 000. DNA polymerase β is not inhibited by sulphydryl-group reagents. The enzyme efficiently copies deoxyribonucleic acid polymers and, to a lesser extent, ribonucleic acid polymers. The cellular function of this form of DNA polymerase may involve repair rather than replication of DNA (Chang and Bollum, 1972). DNA polymerase β probably corresponds to the low molecular weight form of DNA polymerase described by Baril and Laszlo (1970). DNA polymerases α and β have been shown to be immunologically distinct (Spadari *et al.*, 1974).

DNA polymerase γ has a molecular weight of about 110 000 and is present in both cytoplasmic and nuclear fractions. The enzyme requires sulphydryl groups for activity and is characterised by very low Michaelis constants for the deoxyribonucleoside triphosphates, which are approximately an order of magnitude lower than those for the polymerase α and β forms (Wintersberger, 1977).

Mitochondrial DNA polymerase is restricted to the mitochondrial fraction in cells and uses endogenous mitochondrial DNA as a template. The active enzyme from HeLa cells appears to be a dimer of polypeptides with molecular weights of 45 000 and 60 000.

Insight into the roles of the various cellular DNA polymerases in eukaryotes has been sought by analysing the levels of the various enzyme forms in non-replicating and replicating cells.

DNA polymerase from foetal rat liver has been shown to be 15-fold higher than in adult tissue (Ferdinandus *et al.*, 1971). Enzyme activity

decreases after birth, with adult levels being attained within eight weeks (Yeoh and Oliver, 1972). The elevated DNA polymerase activity in foetal liver has been shown to be due to increased levels of the higher molecular weight (polymerase α) form of the enzyme. The levels of the lower molecular weight form of DNA polymerase (polymerase β) are similar before and after birth. The latter becomes the predominant form of the enzyme in differentiated rat liver (Ove *et al.*, 1969a). Similar findings have been reported for rat foetal cardiac muscle (Claycomb, 1975) and cerebellar tissue (Chiu and Sung, 1972). It appears that synthesis of the high molecular weight form of DNA polymerase may be repressed in adult rat liver, only the low molecular weight form of the enzyme being synthesised in this differentiated tissue (Ove *et al.*, 1970).

Following partial hepatectomy, the level of cytosol DNA polymerase activity shows a good correlation with replicative DNA synthesis during the first 24 hours of rat liver regeneration (Bollum and Potter, 1959). This increased DNA polymerase activity is due mainly to an approximately 14-fold increase in the high molecular weight form of the enzyme, which has been shown to have peak activity 48 hours after partial hepatectomy (Iwamura *et al.*, 1968). Levels of activity of the low molecular weight form of DNA polymerase only increased about 4-fold in rat regenerating liver. On the other hand Ove *et al.* (1969a) and Ove *et al.* (1970) have claimed that in rat regenerating liver, only the low molecular weight form of the enzyme shows increased activity, and that elevated levels of the high molecular weight DNA polymerase are characteristic of only embryonic and neoplastic rat liver tissues.

Activity of the high molecular weight form of DNA polymerase has been shown to increase significantly relative to the levels in corresponding normal or host tissues, in slow-growing rat renal tumours, in

human carcinomas of the colon and breast, and in melanomas (Laszlo *et al.* (1969). Furthermore, this form of the enzyme shows increases which correlate with tumour growth rate in the Morris series of transplantable rat hepatomas (Baril and Laszlo, 1970; Ove *et al.*, 1969b). Levels of the low molecular weight form of DNA polymerase also appear to be increased in these rat hepatomas and human carcinomas and melanomas, although the increases are small relative to those of the high molecular weight form of the enzyme.

There appear to be multiple forms of DNA polymerase in the nuclear fraction of Human KB cells, one of which appears to be very similar to DNA polymerase α , and to show good correlation between levels of activity and the rate of cellular division (Sedwick *et al.*, 1972; Wang *et al.*, 1975). Elevated activity of a high molecular weight DNA polymerase, which is restricted to the nuclear fraction of cells from human bronchogenic sarcoma, mammary adenocarcinoma and caecal carcinoma, has been reported by De Philip *et al.* (1977). The activity of this tumour-associated form of nuclear DNA polymerase is inhibited by cytosine-arabinoside triphosphate (Ara-CTP) and it appears to be the form of the enzyme responsible for DNA replication. In the corresponding normal tissue there appears to be only a low molecular weight form of the enzyme which is not affected by inhibitors of DNA replication.

Increases in activity which are unique to the high molecular weight form of DNA polymerase have been reported in mouse spleen cells stimulated with erythropoietin (Roodman *et al.*, 1976), cultured ascites cells (Marujama and Bollum, 1976), and in phytohaemagglutinin-stimulated human peripheral lymphocytes (Loeb *et al.*, 1970).

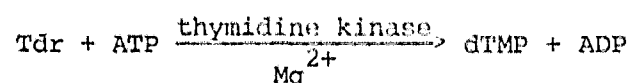
In general, in all cells and tissues studied, the increased DNA polymerase activity during DNA synthesis has been associated with increased

levels of DNA polymerase α activity. More recently it has been suggested that the activity of DNA polymerase γ is also increased during the replication process (Wintersberger, 1977). Levels of DNA polymerase β have been shown to remain fairly constant throughout the cell-cycle and to be comparable in dividing and non-dividing cells. Slight increases in activity of this form have, however, been reported in some cases, as discussed on page 25, and the participation of DNA polymerase β in the processes of cellular replication may not, at this stage, be excluded (Wintersberger, 1977).

DNA polymerase activity in the serum, and increased activity in host liver, have been reported for rats bearing Yoshida sarcoma tumours (Shirasaka and Fujii, 1975). These authors have suggested that the increased activity in host liver is due to some serum-mediated stimulatory factor.

Thymidine Kinase (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.75)

Thymidine kinase catalyses the irreversible phosphorylation of thymidine to deoxythymidine 5'-monophosphate in a reaction which requires ATP as a phosphate donor and the presence of Mg^{2+} , as shown below:



Although Tdr kinase has been assigned a salvage role in DNA synthesis, it shows marked substrate specificity (only Tdr, Udr and the 5'-halo derivatives of Udr may serve as substrates), and is subject to allosteric regulation and feedback control, as discussed on pages 51 and 52. Because of the importance of dTMP in DNA synthesis, and its ability to recycle Tdr towards DNA synthesis, Weber (1977a) has suggested that Tdr kinase is a key enzyme of DNA synthesis.

Multiple forms of thymidine kinase with differing properties have

been reported from various sources: viral (Cheng and Ostrander, 1976; Kit *et al.*, 1974), avian (Kit *et al.*, 1975; Smith and Eakin, 1975), and mammalian (Berk and Clayton, 1973; Eker, 1966; Ohashi and Taguchi, 1976; Adelstein *et al.*, 1971; Hashimoto *et al.*, 1969; Okuda *et al.*, 1972; Machovich and Greengard, 1972; Bresnick and Thompson, 1965; Lee and Cheng, 1976 a,b; Salser and Balis, 1974; Taylor *et al.*, 1972; Gordon *et al.*, 1968; Stafford and Jones, 1972).

There is evidence in favour of separate cytoplasmic and mitochondrial forms of Tdr kinase activity in eukaryotic cells.

A mitochondrial form of Tdr kinase which is specific for the synthesis of mitochondrial DNA, and shows fairly constant activity throughout the life of the cell, has been described in mouse L cells (Berk and Clayton, 1973), and mouse liver tissue (Adelstein *et al.*, 1971).

The cytoplasmic forms of Tdr kinase show decreasing activity with increasing cellular differentiation, and are responsible for the observed differences in enzyme activity between normal and regenerating or tumorous liver tissues. Levels of cytoplasmic Tdr kinase are high at birth but have been shown to decrease subsequently (Köhler, 1972). This decrease in activity after birth has been shown to correlate with a loss of erythropoietic function (Machovich and Greengard, 1972).

In mouse liver, partial hepatectomy causes an increase in the activity of the cytoplasmic form of Tdr kinase which is directly proportional to the rate of incorporation of Tdr into DNA (Adelstein *et al.*, 1971). About 90% of the *in vivo* Tdr kinase activity in cultured mouse spleen cells is associated with a labile cytoplasmic form of the enzyme which shows increased activity following erythropoietin stimulation, correlating with an increased rate of incorporation of Tdr into DNA (Roodman *et al.*, 1976).

Foetal Tdr kinase and the enzyme form found in cancerous human cells may represent genetically distinct forms of the enzyme (Stafford and Jones, 1972), or they may be identical to the cytoplasmic form of the enzyme.

The increases in Tdr kinase activity in rat regenerating liver (Adelstein *et al.*, 1971), in transformed human lymphocytes (Loeb *et al.*, 1970), and in human Chang liver cells (Eker, 1966), are sensitive to Actinomycin D inhibition, suggesting that these increases are dependent upon the process of transcription and probably involve *de novo* protein synthesis.

Tdr kinase activity from rat liver has been shown to exhibit marked daily oscillations under controlled light and feeding conditions with maximum activity occurring at the end of a 'dark' period (Hopkins *et al.*, 1973). Following partial hepatectomy an initial peak of Tdr kinase activity and DNA synthesis occurred at 20-23 hours after surgery. Thereafter a regular light-dark oscillatory pattern, resembling that found in control animals, was noted.

Thymidine kinase generally shows correlation of activity with cellular growth-rate or proliferative capacity (Kit, 1976). This is supported by the finding that Tdr kinase activity is high in 19 day old foetal rat liver and subsequently decreases sharply before term (Köhler, 1972). Following birth there is a more gradual decrease in activity over a period of about 5 weeks, until the low levels characteristic of differentiated adult rat liver are reached (Klemperer and Haynes, 1968). The activity of Tdr kinase shows a correlation with growth-rate in rat transplantable hepatomas (Bukovsky and Roth, 1963; Sneider *et al.*, 1969; and Weber, 1974), and in spontaneous or chemically induced transplantable hepatomas in mice (Bresnick *et al.*, 1971). Tdr kinase in

rapidly-growing Morris transplantable rat hepatomas and Novikoff rat hepatomas is respectively 100-fold and 140-fold higher than in normal rat liver (Weber, 1974). Activities of Tdr kinase have been shown to be relatively higher in human glandular carcinomas of the caecum, rectum, breast; squamous cell carcinomas of the tonsil, larynx and tongue, and in a uterine adenocarcinoma, than in the corresponding normal or host tissues (Gordon *et al.*, 1968).

In contrast to the above findings of increased Tdr kinase activity in rat and mouse regenerating and tumorous liver, the levels of the enzyme activity in a series of slow-growing transplantable rat renal tumours were slightly lower than in rat normal kidney tissue (Laszlo *et al.*, 1969). This finding correlates with the failure of these renal tumours to show increased incorporation of Tdr into DNA. Similarly levels of Tdr kinase activity in human carcinomatous lung and hyper-nephroma tissues have been shown to have, respectively, only 75% and 50% of the activities measured in the corresponding normal tissues (Gordon *et al.*, 1968). In human colonic cancer Tdr kinase activity is comparable with that in control human colon (Laszlo *et al.*, 1969).

Rats bearing solid Yoshida sarcomas show increased levels of serum Tdr kinase. Shirasaka and Fujii (1975) have demonstrated that the serum Tdr kinase resembles that found in the sarcoma. Levels of the Tdr kinase form found in host rat liver are also increased. These findings may represent a leaking into the blood stream of the tumour form of Tdr kinase and, in addition, the release by the tumour of some factor, transported in the serum, which causes increased levels of activity of the form of the enzyme characteristic of host rat livers. Recent work on serum Tdr kinase levels in tumour-bearing animals (Shirasaka and Fujii, 1975; Taylor *et al.*, 1976) suggests that serum analysis for Tdr

kinases characteristic of tumours may serve as a useful technique in the detection of cancer and in the monitoring of response to therapy.

Thymidine kinase serves as a useful tool in *in vitro* studies for the comparison of the rate of DNA synthesis in normal and cancerous cells, and in the investigation of therapeutic regimes, by acting as an entry point for the incorporation of ^3H -Tdr into dTTP. Some forms of chemotherapy (eg. the cytostatic agents 5-fluoro-uracil or 5-fluoro-deoxy-uridine) may depend on the presence of Tdr kinase activity for their conversion to metabolically active forms.

Control of the Key Enzymes of Pyrimidine and DNA Synthesis

The pathways leading to DNA synthesis appear to be subject to control by the deoxynucleotide dTTP. Levels of dTTP have been shown to regulate the activities of ribonucleotide reductase (Murphree *et al.*, 1968), dCMP deaminase (Henderson and Paterson, 1973, p.236), dTMP kinase (Eker, 1968) and Tdr kinase (Klemperer and Haynes, 1968). The activities of these four enzymes have also been shown to be affected by dCTP, but to a lesser extent. The activity of dCMP deaminase is elevated prior to dTMP synthetase and dTMP kinase in rat regenerating liver. It has been suggested that the induction of dCMP deaminase leads to increased levels of dUMP, which in turn may activate and/or induce dTMP synthetase, leading to increased levels of dTMP, which may induce dTMP kinase activity (Maley and Maley, 1960).

The smooth membrane fraction isolated from the post-microsomal supernatant of rat regenerating liver and Novikoff hepatoma has been shown to contain high molecular weight DNA polymerase, ribonucleotide reductase, thymidine kinase, and possibly thymidylate synthetase, which might favour a concerted action by these enzymes in DNA replication (Baril *et al.*, 1972). Localisation of these membrane-associated enzymes

of DNA synthesis on or near the nuclear membrane would allow control of DNA synthesis due to cellular compartmentation, with possible migration of the replicative form of DNA polymerase into the nucleus during periods of DNA synthesis (Iwamura *et al.*, 1968; Baril and Laszlo, 1970; Baril *et al.*, 1972).

PURIFICATION AND PROPERTIES OF THYMIDINE KINASE

Studies on the Purification and Stability of Thymidine Kinase

Purification

The purification and stabilisation of Tdr kinases from various sources are summarised in Table 1. Thymidine kinases from most sources studied are generally unstable, and this has limited the extent to which the enzyme has been purified.

Tdr kinase has been partially purified from animal tumours such as Novikoff ascites hepatoma, McCoy MDAB hepatoma and Walker carcinosarcoma using techniques which include high-speed centrifugation, ammonium sulphate precipitation (0-30%), heating at 60°C for 5 minutes, and negative adsorption onto calcium phosphate gel (Bresnick and Thompson, 1965). Yields with these techniques ranged from 25-35%, and purification factors of about 100-fold were obtained. Attempts by Bresnick and Thompson to purify the enzyme further by ion exchange chromatography on DEAE-cellulose were unsuccessful, as the enzyme lost considerable activity. This procedure was slightly modified by using heat fractionation at 50°C for the purification of Tdr kinase from *E. coli* cells and rat regenerating liver (Bresnick *et al.*, 1970). A purification of 300-fold was achieved for the enzyme from rat regenerating liver, with a yield of 30%.

The purification procedure of Bresnick and Thompson (1970) for rat regenerating liver Tdr kinase has been further developed by Kizer and Holman (1974). Partially purified Tdr kinase was found to adsorb to hydroxy-

TABLE 1
SUMMARY OF PURIFICATION AND STABILIZATION METHODS OF HEPATOCYTAL ESTERASE

Source	Purification Method	Purification	Yield	Stabilization and Storage	Reference
<i>E. coli</i> cells	Preparative PAGE electrophoresis	551 x	14	+ mercaptoethanol	Voytek et al., 1971
<i>E. coli</i> cells	Affinity chromatography, streptomycin affinity fractionation, heat treatment at 70°C	1 470	50%	+ dithiothreitol	Boade & Leane, 1971
<i>E. coli</i> cells and Rat regenerating liver	Streptomycin fractionation, heating at 50°C, negative column phosphate gel adsorption	300 x	50%	+ mercaptoethanol	Bresnick et al., 1970
Rat regenerating liver	Heat treatment (+ATP), streptomycin fractionation, gel filtration on Sephadex G-200	80	14%	Very labile, required ATP	Wilkinson & Wanka, 1971
Rat regenerating liver	As above, Bresnick et al., 1970 plus positive gel adsorption (+ATP), DEAE-cellulose chromatography and column phosphate column	2 000 x	5%	+ mercaptoethanol	Aizer & Holman, 1974
Developing rat liver	100 000 x g supernatant	-	-	ATP required to stabilize enzyme during passage through Sephadex G-200	Menqer & Haynes, 1963
Calf thymus	Fractionation using streptomycin, (NH ₄) ₂ SO ₄ , and phosphate, and gel filtration	150 x	50%	Required high protein, glycerol, and potassium, stored at -20°C	Her & Menqer, 1971
Yoshida sarcoma (rat)	Fractionation using streptomycin, (NH ₄) ₂ SO ₄ , and differential centrifugation	Peak I 1 500 x Peak II -	18% 8%	-	Fujii et al., 1972
Novikoff ascites tumour	Anionic sulphate fractionation, heating at 70°C and negative column phosphate adsorption	120 x	35%	-	Hashimoto et al., 1972
McKay rat hepatoma	-	50 x	27%	-	Bresnick & Thompson, 1966
Walker rat carcinosarcoma	-	100 x	25%	-	-
Human adult liver	100 000 x g supernatant, (NH ₄) ₂ SO ₄ fractionation	-	50-60%	stored in glycerol (50%) at -20°C + mercaptoethanol	Taylor et al., 1972
Human adrenal gland	Fractionation using (NH ₄) ₂ SO ₄ and chromatography on DEAE-cellulose	10 x	18%	-	-
Human hyperplastic adrenal gland	-	18 x	22%	-	Nowata et al., 1976
Human adrenocortical carcinoma	-	Peak I 10 x Peak II 6 x	25%	-	-
Human normal and Ca colon	Fractionation using (NH ₄) ₂ SO ₄ , heating at 70°C, negative column phosphate gel adsorption, gel filtration on Sephadex G-200, and DEAE-cellulose chromatography	2 200 x	6.4%	-	Salser & Ballis, 1976
Human myelocytic leukemia blast cells	cytochrome c, plasma, and differential affinity chromatography	Peak I 2 410 x Peak II 1 651 x	50% 9%	Unstable during concentration	Lee & Cheng, 1976a & 1976b

PAGE = Polyacrylamide gel electrophoresis

apatite in the presence of ATP and was subsequently eluted using an ionic gradient. A DEAE-Sephadex step was also included in the purification procedure. Using these additional purification steps they obtained a 2 600-fold purified enzyme preparation, with a yield of 5%.

Rat regenerating liver Tdr kinase has also been purified by Walboomers and Wanka (1974). These authors observed that the loss of enzyme activity during gel-filtration could be reduced in the presence of ATP. They obtained a 14% yield with an 80-fold purification.

Tdr kinases from rat Yoshida sarcoma have been purified by fractionation using streptomycin and ammonium sulphate, and by chromatography on hydroxylapatite and DEAE-cellulose (Fujii *et al.*, 1972; Hashimoto *et al.*, 1972). Two fractions of Tdr kinase were separated by DEAE chromatography. The Peak I form which was eluted by 50mM NaCl in Tris-HCl buffer, pH 8.0, was purified 1 500-fold, the yield being 13%. The Peak II form was eluted by 400 mM NaCl in Tris-HCl buffer, pH 8.0, and was purified at least 25-fold, the yield being 8.3%.

Calf thymus Tdr kinase has been purified 130-fold with a 50% yield, using techniques which included fractionations with streptomycin sulphate, ammonium sulphate and protamine sulphate, followed by gel-filtration on Sephadex G-100 (Her and Momparler, 1971).

Human colonic Tdr kinase has been partially purified by means of high-speed centrifugation, ammonium sulphate precipitation, heating at 50°C in the presence of 1mM Tdr, negative adsorption on calcium phosphate gel, and chromatography on Sephadex G-200, Bio-gel, and DEAE cellulose (Salser and Balis, 1976). The final preparation gave a yield of 6.4% and an overall purification of 2 200-fold.

Tdr kinase has been partially purified from human adrenocortical carcinoma and from normal and hyperplastic adrenal glands (Nawata *et al.*,

1976). The techniques used included 0-30% ammonium sulphate fractionation and chromatography on DEAE cellulose. Two fractions of Tdr kinase activity were obtained from human adrenocortical carcinomas on DEAE chromatography: Peak I was eluted with 100mM NaCl in Tris-HCl buffer, pH 7.5, and was purified about 10-fold; Peak II was eluted with 400mM NaCl in Tris-HCl, pH 7.5, and was purified about 6-fold. The overall yield for Peaks I and II was 23%. A single Tdr kinase form from hyperplastic adrenal gland, corresponding to a Peak I fraction, was purified 18-fold, the yield being 22%. A single form of Tdr kinase, also corresponding to the tumour Peak I fraction, was purified about 10-fold from human normal adrenal gland. The yield was 18%.

Tdr kinases from human foetal and adult liver tissues, and from human HeLa and KB cells have been partially purified using centrifugation at 105 000 x g for 90 minutes, followed by precipitation between 0 and 40% ammonium sulphate saturation (Taylor *et al.*, 1972; Stafford and Jones, 1972). The resuspended precipitates were stored in 50% glycerol at -20°C. About 50-60% of the activity measured in the crude homogenate was recovered but the purification achieved was not given. These preparations were used for physical and regulatory studies.

An affinity chromatography purification method, using the substrate 5'-amino-5'-deoxythymidine as a ligand, has been described by Rohde and Lezius (1971). Using this technique they achieved a 190-fold purification of *E. coli* B Tdr kinase, with a 90% yield, in a single purification step. When this step was combined with streptomycin and ammonium sulphate precipitation and heating of the enzyme preparation for 5 minutes at 70°C, an overall purification 1 220-fold, with a 50% yield, was obtained. Recently a 370-fold single-step purification of Tdr kinase from *E. coli*, using affinity chromatography, has been described (Rohde, 1977). The

efficiency of affinity chromatography as a purification technique is well illustrated by comparison of these results with those of Voytek *et al.* (1971) who used preparative polyacrylamide gel electrophoresis to purify *E. coli* Tdr kinase. These authors obtained an overall purification of 531-fold but the yield using this method was only about 1%.

The ligand described by Rohde and Lezius was found to be unsuitable for the purification of Tdr kinase from mouse 180 sarcoma cells (Cheng and Prusoff, 1974). A thymidine-3'-(p-aminophenyl phosphate) ligand, developed by Kowal and Markus (1975), has been used by these authors to achieve a 109-fold purification of Tdr kinase from mouse gut. Subsequently affinity chromatography using this ligand has been coupled with fractionation procedures using streptomycin and ammonium sulphate to purify and separate two Tdr kinase forms from blast cells of human patients with acute myelocytic leukaemia (Lee and Cheng, 1976a and b). The cytoplasmic form of the enzyme was purified 2 416-fold, with a 36% yield, while mitochondrial Tdr kinase was purified 1 634-fold, with a yield of 9%.

Stability

Human normal or neoplastic colon, laryngeal, mammary gland, pulmonary, and renal tissues showed no significant loss of Tdr kinase activity when stored at -70°C for longer than 1 year (Gordon *et al.*, 1968). Similar findings have been reported for rat hepatoma tissue stored at -17°C (Lukovsky and Roth, 1963). The enzyme from animal tissues appears, however, to be labile during prolonged purification procedures (Bresnick and Thompson, 1965),

Enzyme activity may be stabilised by the presence of thymidine (Bresnick and Thompson, 1965; Olsen and Harris, 1975; Cheng and Prusoff, 1974); in the presence of 20-30% glycerol (Her and Momparler, 1971); or when purified or partially-purified extracts are stored in 50% glycerol

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at -20°C (Taylor *et al.*, 1972). Thymidine kinase from foetal rat liver has been stabilised during gel-filtration by the presence of 1mM ATP (Klemperer and Haynes, 1968). In the absence of ATP 75% of the activity of the enzyme was lost during filtration on a Sephadex G-25 column, whereas this loss was reduced to 10-20% in the presence of ATP. Stabilisation of Tdr kinase in the presence of ATP has also been reported by Walboomers and Wanka (1974) and by Cheng and Prusoff (1974). The finding that Tdr kinase would only adsorb onto calcium phosphate gel in the presence of ATP (Kizer and Holman, 1974) suggests that the presence of ATP may lead to a conformational change.

In addition to ATP or Tdr, the enzyme forms from human leukaemic blast cells required the presence of dithiothreitol for stability (Lee and Cheng, 1976b). β -mercaptoethanol has been used to stabilise Tdr kinase preparations from calf thymus (Her and Momparler, 1971); rat and human intestines (Salser and Balis, 1973); and from human adult and foetal liver (Taylor *et al.*, 1972). Tdr kinases from Novikoff ascites hepatomas, MDAB hepatomas and Walker carcinosarcomas are inactivated by sulphhydryl group inhibitors such as mercury or p -hydroxymercuribenzoate (Bresnick and Thompson, 1965). Similar findings have been reported for rat Yoshida sarcoma Tdr kinase (Hashimoto *et al.*, 1972). However the presence of reduced thiols is not always advantageous for the stabilisation of Tdr kinases, since mercaptoethanol has been reported to increase the rate of inactivation of a Tdr kinase preparation from mouse 180 sarcoma cells (Cheng and Prusoff, 1974).

Physical Properties of Thymidine Kinase

Studies of the physical properties of Tdr kinases are summarised in Table 2.

TABLE 2

SUMMARY OF STUDIES ON PHYSICAL PROPERTIES OF THYMIDINE KINASES

Source	Molecular Weight	Multiple Enzyme Form on: Electrophoresis or BLA	pH Optimum	Temperature Stability	Effects of Salts or Metal Ions	Reference
<i>E. coli</i> cells	3.4S (+dCMP-5.3S)	Single band -	6-8	-	Mg ²⁺ not effective	Voytek <i>et al.</i> , 1971
<i>E. coli</i> cells	46 500	Single band -	-	-	Neutral salts + dissociation	Bohle & Lezins, 1971
<i>E. coli</i> cells	49 300(5.6S)	Single band(R _m ² =0.71) -	-	-	-	Bresnick <i>et al.</i> , 1970
Rat regenerating liver	80 600(5.0S)	Single band(R _m ² =0.1) -	-	-	-	Bresnick <i>et al.</i> , 1970
Rat regenerating liver	130 000	-	-	Stabilised by ATP or Tdr	Activated by Mg ²⁺	Walboomers & Wanka, 1974
Rat regenerating liver	69 000	Eight bands on PAGE ¹ -	8.0	-	-	Kizer & Holman, 1974
Foetal (F) & adult(A) rat liver	-	-	8.0	More stable than A. Both stabilised by ATP	-	Klemperer & Haynes, 1968
Foetal(F) & adult(A) rat cardiac muscle	69 000	-	9.0	-	Requires Mg ²⁺ at Mg:ATP ratio of 0.5:1	Gillette & Claycomb, 1974
Calf thymus	55 000	-	8.5	Inactivation decreased by Mg:ATP ratio of products, substrates and inhibitors	-	Her & Mampieri, 1971
Yoshida sarcoma - Peak I	70 000	-	8.0	-	-	Fujii <i>et al.</i> , 1972
Yoshida sarcoma - Peak II	120 000	-	8.0	-	-	Hashimoto <i>et al.</i> , 1972
Yoshida sarcoma cells	-	Two bands, low R _m 's	-	-	-	Hamazaki, 1973
Rat regenerating liver	-	Single low R _m band	-	-	-	Okada <i>et al.</i> , 1972 & Hashimoto <i>et al.</i> , 1969
Rat liver	-	Single low R _m band	-	-	-	
Novikoff ascites tumour	670 000	-	8.0	-	Require Mg ²⁺	Bresnick & Thompson, 1965
McCoy MEAB rat hepatoma		-		-		
Walker rat carcinosarcoma		-		-		
				Tdr prevents inactivation due to heating at 60°C for 5 minutes		
Mouse ascites carcinoma cells	-	-	-	Stabilised by ATP	Requires Mg ²⁺ (Mg ²⁺ & ATP)	Cheng & Prusoff, 1974

(Table 2 continued)

(Table 2 continued)

Source	Molecular Weight	Multiple Enzyme forms on: Elec.rophoresis or D.M.	pH Optimum	Temperature Stability	Effects of Salts or Metal Ions	Reference
Human adult liver	-	Rapidly migrating form only	-	Bimodal 7.0-7.5	Lost 10% activity on heating 5' at 45°C	-
Human foetal liver	-	Two slow bands & 1 rapidly migrating band	-	7.0-7.2	Lost >50% activity on heating 5' at 45°C	-
Human HeLa & KB cell-lines	-	-	-	7.0-7.2	-	-
Human renal, bladder & muscle tumours	-	Two slow bands & 1 rapidly migrating band	-	7.0-7.2	Lost >50% activity on heating 5' at 45°C	-
Human adrenal gland	-	-	Peak I (100mM NaCl)	7.5	Lost 10% activity on heating 5' at 45°C	Requires Mg ²⁺
Human hyperplastic adrenal gland	-	-	Peak I (100mM NaCl)	7.5	Lost >50% activity on heating 5' at 45°C	-
Human adrenocortical carcinomas	-	-	Peak I (100mM NaCl) Peak II (400mM NaCl)	7.5	Lost >50% activity on heating 5' at 45°C	Requires Mg ²⁺
Human myelocytic leukaemia blast cells	cyto-plasmic 80 000 mitochondrial 70 000	R _m = 0.1 R _m = 0.6	-	7.6-7.8	Stable 40' at 25-40°C	Inhibited by (NH ₄) ₂ SO ₄ ; Mg:ATP ratio of 1:1
				7.4-7.8	Stable 40' at 25-40°C	Inhibited by (NH ₄) ₂ SO ₄ ; Mg:ATP ratio of 1:1

1. PAGE - Polyacrylamide gel electrophoresis

2. R_m - the R_m of a protein in solution in its mobility relative to that of the marker dye front.

Molecular Weight

A molecular weight of 670 000 for Tdr kinases from Novikoff ascites and MDAB hepatomas and Walker carcinosarcomas has been estimated using gel-filtration on Sephadex G-200 (Bresnick and Thompson, 1965). The molecular weight of the enzyme from calf thymus appears to be 55 000, using both gel-filtration and sucrose density gradient determinations (Her and Momparler, 1971). Molecular weights of 69 000 and 81 000 for rat regenerating liver Tdr kinase have been obtained by, respectively, Kizer and Holman (1974) and Bresnick *et al.* (1970), using sucrose density gradients. Both of these values are lower than the molecular weight of 130 000 obtained by Walboomers and Wanka (1974) for the enzyme from rat regenerating liver using gel-filtration on Sephadex G-200, but are similar to the molecular weight of 69 000 determined for rat cardiac muscle Tdr kinase using a density gradient (Gillette and Claycomb, 1974). The molecular weights of the mitochondrial and cytoplasmic forms of Tdr kinase from human myelocytic leukaemia blast cells have been shown to be 70 000 and 90 000 respectively, using gel-filtration (Lee and Cheng, 1976b).

Deoxyribonucleotides affect the state of aggregation of Tdr kinase from *E. coli* (Bresnick *et al.*, 1970). Using sucrose density gradients, the enzyme was shown to have a sedimentation constant of 3.6S in the presence of ATP or the activator dCDP. dTTP increased the sedimentation constant to 6.0S. Similar results have been reported by Voytek *et al.* (1971), who have shown that *E. coli* cells have a sedimentation constant of 3.4S, which is increased to 5.3S in the presence of dCTP. The sedimentation constant for Tdr kinase from rat regenerating liver is, however, not affected by the presence of dCDP, ATP or dTTP (Bresnick *et al.*, 1970). It may be that *E. coli* Tdr kinase undergoes polymerisation from a mono-

meric state to an oligomeric state in the presence of these nucleotide effectors, whereas the enzyme from rat regenerating liver already exists as an oligomer.

Aggregated and disaggregated forms of Tdr kinase have been noted for the Walker carcinosarcoma enzyme (Bresnick *et al.*, 1966). At low ionic strength the aggregated form was found to have a molecular weight of 600 000, whereas a molecular weight of 110 000 was obtained at higher ionic strength (i.e. for the disaggregated form). These molecular weights were determined using gel-filtration on Sephadex G-200. Similar results have been reported for Yoshida sarcoma Tdr kinase (Fujii *et al.*, 1972). The possibility that the modifier causing aggregation might be RNA or might contain RNA is suggested by the finding that the addition of RNase to a crude Tdr kinase preparation led to the appearance of the disaggregated form of the enzyme (Okula *et al.*, 1972; Hamazaki, 1973). This effect was not observed when DNase was added to the Tdr kinase preparation. In addition, reagents which affect protein quaternary structure, such as sodium dodecyl sulphate, inhibit Tdr kinases from Novikoff ascites and MDAB rat hepatomas and rat Walker carcinosarcomas (Bresnick and Thompson, 1965).

Temperature Stability

The Tdr kinase form characteristic of human foetal liver is more labile than that from the adult tissue (Taylor *et al.*, 1972). Foetal Tdr kinase was found to lose 50% of its activity within 5 minutes when incubated at 45°C, while the adult form showed only a 10% decrease in activity when incubated under the same conditions. Furthermore, on incubating human adult and foetal liver forms of Tdr kinase for 5 minutes at increasing temperatures, it was found that 50% of the foetal enzyme activity was lost between 35°C and 45°C, while the same loss of activity of the adult form

of the enzyme required incubation at 45-50°C. Tdr kinases from HeLa and human KB cell-lines have been shown to have thermostabilities similar to that found in human foetal liver (Taylor *et al.*, 1972). Tdr kinases from human normal adrenal gland and adrenocortical carcinomas show temperature stability differences which are analogous to those shown by adult human liver and foetal liver or tumour cell Tdr kinase preparations (Nawata *et al.*, 1976).

Although the heat-inactivation curves of normal and cancerous human colon Tdr kinases differed, both were found to be biphasic, suggesting that normal and cancerous colon tissues each contain different forms of the enzyme, or that there is a heat-labile effector present in both the normal and tumorous forms of the enzyme (Salser and Balis, 1974).

In contrast to the relatively labile Tdr kinase forms in human foetal and cancerous tissues, the mitochondrial and cytoplasmic forms of the enzymes from human leukaemic blast cells appear to be stable at temperatures of 25-40°C for 40 minutes (Lee and Cheng, 1976b).

Various substrates, products, and inhibitors of Tdr kinase (such as Tdr, Udr, ATP, dATP, dTMP, dTDP and dTTP) can reduce or prevent heat inactivation of calf thymus Tdr kinase (Her and Momparler, 1971) and the enzyme from rat regenerating liver (Walboomers and Wanka, 1974). In HeLa TK⁻ cells infected with Herpes simplex viruses types I and II, which induce nuclear and cytoplasmic Tdr kinases respectively, Cheng and Ostrander (1976) have shown that Mg-ATP preferentially stabilises the induced HeLa type II Tdr kinase, whereas the presence of Tdr leads to preferential stabilisation of the type I-induced form of the enzyme.

pH Optimum

Thymidine kinase from human foetal liver shows optimum activity at pH 7.0-7.2 whereas the adult form of the enzyme has a bimodal pH

optimum with peaks at 7.0 and 8.5 (Taylor *et al.*, 1972). The pH optima of human KB and HeLa cells appear to be similar to that of human foetal liver (Stafford and Jones, 1972). The mitochondrial and cytoplasmic forms of human leukaemic blast cell Tdr kinase have pH optima of 7.4-7.8 and 7.6-7.8 respectively (Lee and Cheng, 1976b). The Tdr kinases in human adrenal glands and human adrenocortical carcinomas show similar broad pH optima in the range of pH 7.5 to 8.8, with peaks at about pH 7.5 (Nawata *et al.*, 1976).

pH Optima of 8.0 and 9.0 respectively, have been reported for Tdr kinases from Walker carcinosarcoma (Bresnick and Thompson, 1965) and from rat cardiac muscle (Gillette and Claycomb, 1974). Foetal and adult rat liver and rat regenerating liver Tdr kinases have pH optima of 8.0 (Klemperer and Haynes, 1968; Klier and Holman, 1974).

Divalent Metal Ion Requirements

Thymidine kinase, like other kinases, requires the presence of divalent metal ions for activity. The enzyme from Walker carcinosarcoma has been reported to show a specific requirement for Mg^{2+} , other divalent cations producing inhibition of the enzyme (Bresnick and Thompson, 1965). The requirement for Mg^{2+} has been confirmed for Tdr kinase from mouse sarcoma 180 cells, although the enzyme is inhibited if the concentration of Mg^{2+} exceeds that of ATP (Cheng and Prusoff, 1974). Similarly both mitochondrial and cytoplasmic Tdr kinases from human leukaemic blast cells show optimum activity at Mg:ATP ratios of 1:1 (Lee and Cheng, 1976c). Inhibition was observed in the presence of excess ATP. In contrast to the findings noted above for mouse sarcoma 180 Tdr kinase, excess Mg^{2+} does not appear to inhibit the activity of the enzyme forms from leukaemic blast cells. Lee and Cheng (1976b) have shown that the leukaemic blast cell forms of Tdr kinase also show activity in the

presence of other divalent metal ions, the preferences of the two forms of the enzyme being as listed below:

Cytoplasmic Tdr kinase: $Mg^{2+} > Ca^{2+} > Mn^{2+} > Cu^{2+} > Fe^{2+} > Zn^{2+}$

Mitochondrial Tdr kinase: $Mg^{2+} > Cu^{2+} > Mn^{2+} > Ca^{2+} > Fe^{2+} > Zn^{2+}$

Using rats with implanted 3'Me-DAB-induced tumours, fed on diets containing high, normal or low Zn^{2+} -supplements, it has been shown that both the Zn^{2+} -supplemented and Zn^{2+} -deficient diets produce decreases in both tumour growth and DNA synthesis relative to the normal diet (Duncan *et al.*, 1974; Duncan and Dreosti, 1975; Duncan and Dreosti, 1976a,b; Prasad, 1977). Similar effects were observed in rats fed on high or low Zn^{2+} -containing diets prior to tumour implantation. These effects have been correlated with decreased Tdr kinase activity (Duncan and Dreosti, 1976a; Prasad *et al.*, 1974; Prasad, 1977). The precise role played by zinc in Tdr kinase activity has not yet been established.

Effects of Salts

The effects of salts on Tdr kinase have been investigated by Cheng and Ostrander (1976), using Tdr kinases induced in HeLa TK⁻ cells by Herpes simplex virus (HSV) types I and II. The type I and type II-induced Tdr kinases were shown to be sensitive to inhibition by ammonium sulphate. Sodium sulphate produced similar inhibition, while sodium chloride had little effect, showing that inhibition is due to the sulphate moiety. The cytoplasmic form of Tdr kinase from human leukaemic blast cells appears to be more sensitive to inhibition by ammonium sulphate than the mitochondrial form of the enzyme (Lee and Cheng, 1976b).

Separation of Multiple Forms of Thymidine Kinase Activity

Electrophoresis

Polyacrylamide gel electrophoresis of Tdr kinase from *E. coli* produced a single band of activity (Voytek *et al.*, 1971), which showed

more rapid anodal migration than the enzyme from rat regenerating liver (Bresnick *et al.*, 1970).

Tdr kinase from an 18cm human foetus exhibited 3 bands of enzyme activity on polyacrylamide gel electrophoresis, with mobilities relative to the marker dye front (R_m) of approximately 0.02, 0.20 and 0.50 (Taylor *et al.*, 1972). These three bands were also observed in a 28cm human foetus. In the latter case, however, there was a marked increase in the relative proportion of the rapidly migrating form of the enzyme. The rapidly migrating Tdr kinase was the only form of the enzyme present in human adult liver. Similar results were obtained using starch-block electrophoresis (Taylor *et al.*, 1972).

The mitochondrial Tdr kinase of HeLa BU 25 cells, which lacks the cytoplasmic form of the enzyme, and of S3 cells showed relative electrophoretic mobilities of 0.4 and 0.6 respectively. Under the same conditions the R_m of the cytoplasmic form of the enzyme from S3 cells was 0.25 (Kit and Leung, 1974). Using polyacrylamide gel electrophoresis of Tdr kinases from blast cells of patients with myelocytic leukaemia, Lee and Cheng (1976b) have shown that, under the conditions used, the cytoplasmic form of the enzyme has an R_m of 0.1, whereas the mitochondrial Tdr kinase has an R_m of 0.6.

Starch-block zone electrophoresis of Tdr kinases from rat bone marrow, spleen and regenerating liver suggests that the enzymes from these tissues are electrophoretically similar, having a very low R_m (Hashimoto *et al.*, 1969). A single band of Tdr kinase activity, showing rapid migration towards the anode, has been observed for the enzyme from foetal rat liver under the conditions used. On starch-block electrophoresis Tdr kinase from human KB cells showed two bands of activity: one remaining at the origin and the other migrating towards the anode (Hashimoto *et al.*, 1969).

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Tdr kinase from an 18cm human foetus exhibited 3 bands of enzyme activity on polyacrylamide gel electrophoresis, with mobilities relative to the marker dye front (R_m) of approximately 0.02, 0.20 and 0.50 (Taylor *et al.*, 1972). These three bands were also observed in a 28cm human foetus. In the latter case, however, there was a marked increase in the relative proportion of the rapidly migrating form of the enzyme. The rapidly migrating Tdr kinase was the only form of the enzyme present in human adult liver. Similar results were obtained using starch-block electrophoresis (Taylor *et al.*, 1972).

The mitochondrial Tdr kinase of HeLa BU 25 cells, which lacks the cytoplasmic form of the enzyme, and of S3 cells showed relative electrophoretic mobilities of 0.4 and 0.6 respectively. Under the same conditions the R_m of the cytoplasmic form of the enzyme from S3 cells was 0.25 (Kit and Leung, 1974). Using polyacrylamide gel electrophoresis of Tdr kinases from blast cells of patients with myelocytic leukaemia, Lee and Cheng (1976b) have shown that, under the conditions used, the cytoplasmic form of the enzyme has an R_m of 0.1, whereas the mitochondrial Tdr kinase has an R_m of 0.6.

Starch-block zone electrophoresis of Tdr kinases from rat bone marrow, spleen and regenerating liver suggests that the enzymes from these tissues are electrophoretically similar, having a very low R_m (Hashimoto *et al.*, 1969). A single band of Tdr kinase activity, showing rapid migration towards the anode, has been observed for the enzyme from foetal rat liver under the conditions used. On starch-block electrophoresis Tdr kinase from human KB cells showed two bands of activity: one remaining at the origin and the other migrating towards the anode (Hashimoto *et al.*, 1969).

Ion-exchange Chromatography

Using DEAE-cellulose ion-exchange chromatography and gradient elution at pH 8.0, two peaks of Tdr kinase activity have been separated from Yoshida sarcoma and human KB cell-lines (Okuda *et al.*, 1972; Hashimoto *et al.*, 1972; Hamazaki, 1973; Fujii *et al.*, 1972). The first peak (Peak I) eluted at 50mM NaCl concentration, whereas elution of the second peak (Peak II) required a 400mM concentration of NaCl for elution. Similarly Tdr kinases in mouse spleen cells infected with Friend virus, and in the livers of rats hosting Yoshida sarcoma tumours, gave 2 peaks of enzyme activity on DEAE cellulose chromatography, whereas the corresponding normal tissues showed only a single peak of enzyme activity corresponding to the Peak I form of Tdr kinase (Ohashi and Taguchi, 1976).

On DEAE chromatography Tdr kinases from rat foetal and regenerating liver tissues gave single peaks which resembled the Peak I forms of the enzyme described for Yoshida sarcoma tumours by Fujii *et al.* (1972).

Two forms of Tdr kinase which are eluted at concentrations of 100mM NaCl (Peak I) and 400mM NaCl (Peak II) have been described for human adrenocortical carcinoma (Nawata *et al.*, 1977). In the corresponding normal adrenal gland only the Peak I form of the enzyme is present.

On injecting rats with the non-carcinogenic haemolytic drug 1-acetyl-2 phenylhydrazine, the Peak II form of Tdr kinase could be detected. For this reason Ohashi and Taguchi (1976) have suggested that the Peak II form of Tdr kinase may not be an exclusively tumour-specific form, but may represent a form which is expressed by the cell genome under conditions of specific stress such as tumorigenesis and anaemia.

Kinetic and Regulatory Properties of Thymidine Kinase

Apparent Michaelis constants (K_m) for ATP and thymidine, for Tdr kinase preparations, as well as studies on phosphate donors and acceptors,

and activation or inhibition by nucleosides and nucleotides, are summarised in Table 3.

Apparent Michaelis Constants

It can be seen from Table 3 that the K_m 's for thymidine and ATP reported for Tdr kinase preparations from eukaryotes are in the ranges 1.2×10^{-6} to 5.7×10^{-5} M, and 5×10^{-5} to 2.6×10^{-3} M, respectively.

The K_m for thymidine for Tdr kinase from *E. coli* is dependent on dCDP (Bresnick et al., 1970). In the presence of dCDP the *E. coli* enzyme showed increased activity a higher V_{max} and a decreased K_m for thymidine which became comparable with that for the enzyme from rat regenerating liver. In contrast to these findings the K_m for rat regenerating liver Tdr kinase was found to be independent of either ATP or dCDP (Bresnick et al., 1970).

Non-linear Lineweaver-Burk plots of velocity versus ATP concentration have been described for Tdr kinases from both *E. coli* cells and from rat regenerating liver (Bresnick et al., 1970). At ATP concentrations below 10^{-3} M, the apparent K_m for ATP for the enzyme from rat regenerating liver was 20mM, whereas this constant decreased to 2.6mM at ATP levels above 10^{-3} M. Similar non-hyperbolic Lineweaver-Burk plots with respect to ATP have been observed for Tdr kinases from rat Walker carcinosarcoma tissue (Bresnick and Thompson, 1965), and for the cytoplasmic form of the enzyme from human leukaemic blast cells (Lee and Cheng, 1976c). The latter authors have noted that the sigmoidal kinetic behaviour of ATP with the cytoplasmic form of human leukaemic blast cell Tdr kinase may be a result of either co-operative or mechanistic effects, or both.

Phosphate Acceptors

Both thymidine and deoxyuridine have been shown to act as phosphate

TABLE 3
SUMMARY OF KINETIC STUDIES ON DEHYDROGENASES

Source	K_m Tdr (M)	K_m AIP (M)	Phosphate Donors	Phosphate Acceptors	Nucleotide Activators	Nucleotide Inhibitors	Reference
<i>E. coli</i> cells	8.5×10^{-5}	-	-	Tdr, Udr & Thio-Udr	dGTP, dATP, dCTP	dTTP (compet)	Voytek et al., 1971
<i>E. coli</i> cells	-	-	-	-	-	Tdr analogues	Bohde & Lohmeyer, 1971
<i>E. coli</i> cells	9.4×10^{-6} (+ dGTP)	4.0×10^{-2}	-	-	-	-	Bresnick et al., 1970
Rat regenerating liver	5.6×10^{-6}	Significant (AIP) 2.5×10^{-2} (AIP) 2.0×10^{-3}	-	-	-	-	Bresnick et al., 1970
Rat regenerating liver	1.2×10^{-6}	3.0×10^{-4}	AIP, dATP only	-	-	dTTP compet w.r.t. AIP but not Tdr	Kallstrom & Winkler, 1974
Rat regenerating liver	3.2×10^{-6}	3.0×10^{-4}	-	-	-	dTTP complex w.r.t. AIP, non-compet w.r.t. Tdr	Aizer & Holman, 1974
Foetal (F) & adult (A) rat liver	F 2.5×10^{-6} A 2.1×10^{-6}	-	-	-	-	dTTP inhibi- tion F greater than A	Kemper & Haynes, 1968
Foetal (F) & adult (A) rat cardiac muscle	1.4×10^{-5}	-	-	-	-	dGTP & dTTP inhibitors of both A & F	Gillette & Chapot, 1974
Calf thymus	5.7×10^{-5}	5.0×10^{-5}	All nucleotides except dTTP	Tdr & Udr	-	dTTP compet w.r.t. Tdr, complex w.r.t. AIP, dATP, dGTP & dCTP	Her & Kemper, 1971
Yoshida sarcoma Peak I	3.3×10^{-5}	8×10^{-5}	AIP & dATP, CTP & GTP	Tdr & Udr	-	dTTP	Fujii et al., 1972
Yoshida sarcoma Peak II	3.4×10^{-5}	7×10^{-5}	AIP & dATP, CTP & GTP	Tdr & Udr	-	dTTP	Hashimoto et al., 1972
Yoshida sarcoma cells	-	-	-	-	c-AMP (Peak II)	c-AMP (Peak I)	Hamada, 1973 Okada et al., 1972 & Hashimoto et al., 1969
Kovikoff ascites rat tumour McCoy MAB rat hepatoma	2.7×10^{-5} x 10^{-6}	Significant 3.4×10^{-4} (AIP)	-	Tdr & Udr & thio-Udr derivatives	-	Br-, F-, I-, Cl-Udr competitive, dTTP complex w.r.t. AIP non-compet w.r.t. Tdr	Bresnick & Thompson, 1965
Walker rat carcinoma sarcoma	9×10^{-6}	1.5×10^{-4}	All nucleotides except dTTP	Tdr & Udr & thio-Udr inhibitors	-	5-NH ₂ , -Br, -Cl- derivatives of Udr	Cheng & Prusoff, 1974
Human adult liver	-	-	AIP, dATP, dGTP, dCTP, dTTP	-	-	dTTP, dGTP & dCTP	Taylor et al., 1972
Human foetal liver	-	-	AIP & dATP	-	-	dTTP, dGTP, no effect due to dCTP	
Human HeLa & KB cell-lines	-	-	-	-	-	-	

(Table 3 continued)

(TABLE 3 continued)

Source	K_m Tdt (M)	K_m ATP (M)	Phosphate Donors	Phosphate Acceptors	Nucleotide Activators	Nucleotide Inhibitors	Reference
Human renal, bladder, uterine & muscle tumours	-	-	-	-	-	dTTP, dTDP; no effect due to dCTP	Stafford & Jones, 1972
Human adrenal gland	25.0×10^{-6}	-	-	-	-	dTTP, dCTP	Nawata et al., 1976
Human hyperplastic adrenal gland	5.1×10^{-6}	-	-	-	-	dTTP	
Human adrenocortical carcinoma	Peak I 5.0×10^{-6}	-	-	-	-	Peak I dTTP	
	Peak II 11.1×10^{-6}	-	-	-	-	Peak II dTTP, dCTP	
Human carcinomas of the caecum, rectum, tongue, larynx, and hypernephroma	(liver) 2.1×10^{-6}	-	-	-	-	dTTP indu- ction in tumours vs. controls	Gordon et al., 1978
Human myelo- cytic leukaemia blast cells	cyto- plasmic 2.6×10^{-6}	cytoplasmic 2.2×10^{-4}	ATP & dATP	Tdt & deoxyrib- osyl derivatives	No effect due to c-MP	dTTP indu- ction with Tdt & ATP	Ito & Chang, 1976 & 1976a
	mito- chond- rial 2.2×10^{-6}	mitochondrial 1.0×10^{-4}	ATP, dATP & CTP	Tdt, dTdt- ribosyl derivatives & CTP	No effect due to c-MP	Also induc- tion by primar- ily analogues	

+ High

+ Low

F = foetus

A = adult

(TABLE 3 continued)

Source	K_m Tdr (M)	K_m AIP (M)	Phosphate Donors	Phosphate Acceptors	Nucleotide Activators	Nucleotide Inhibitors	Reference
Human renal, bladder, uterine & muscle tumors	-	-	-	-	-	dTTP, dUMP; no effect due to dCTP	Stafford & Jones, 1972
Human adrenal gland	25.0×10^{-6}	-	-	-	-	dTTP, dCTP	Nawata et al., 1976
Human hyperplastic adrenal gland	5.1×10^{-6}	-	-	-	-	dTTP	
Human adrenocortical carcinoma	Peak I 5.0×10^{-6} Peak II 11.1×10^{-6}	-	-	-	-	Peak I dTTP Peak II dTTP, dCTP	
Human carcinomas of the caecum, rectum, tongue, larynx, and hypernephrosis	(larynx) 2.1×10^{-6}	-	-	-	-	dTTP inhibi- tion in tumors of controls	Gordon et al., 1968
Human myelo- cytic leukemia	cyto- plasmic 2.6×10^{-6}	Sigmoidal 2.2×10^{-4}	AIP & dATP	Tdr & halo-Tdr derivatives	No effect due to c-MP	dTTP inhibi- tion w.r.t. Tdr & AIP	Lee & Chang, 1976b & 1976c
blast cells	mito- chon- drial 5.2×10^{-6}	1.0×10^{-4}	AIP, dATP & CTP	Tdr, halo-Tdr derivatives & Cdr	No effect due to c-MP	Also inhibi- tion by penan- tine analogues	

+ High

+ Low

F = fetal

A = adult

acceptors in the reactions catalysed by Tdr kinases from rat hepatomas and sarcomas (Bresnick and Thompson, 1965); Yoshida sarcoma cells (Hashimoto *et al.*, 1972) and calf thymus (Her and Momparler, 1971).

The 5-halo derivatives of deoxyuridine, 5-fluoro-deoxyuridine (5-F-Udr), 5-chloro-deoxyuridine (5-Cl-Udr), 5-bromo-deoxyuridine (5-Br-Udr), and 5-iodo-deoxyuridine (5-I-Udr) may act as phosphate acceptors with Tdr kinases from rat Novikoff ascites and MDAB hepatomas and Walker carcinosarcomas (Bresnick and Thompson, 1965). These derivatives of deoxyuridine have been shown to act as competitive inhibitors of the natural substrate, Tdr, in Walker carcinosarcomas in rats (Bresnick and Thompson, 1965). 5-I-Udr and 5-Br-Udr have been shown to be potent inhibitors, with respect to thymidine, of Tdr kinases from human leukaemic blast cells (Lee and Cheng, 1976c). The mitochondrial form of the enzyme was less susceptible to 5-I-Udr and 5-Br-Udr inhibition than the cytoplasmic form. On the other hand 5-F-Udr and deoxyuridine have been shown by these authors to be relatively weak inhibitors of the two enzyme forms. Lee and Cheng obtained inhibitor constants for 5-I-Udr, 5-Br-Udr, 5-F-Udr, and deoxyuridine of 2.4 μ M, 1.8 μ M, 26 μ M and 273 μ M respectively for the cytoplasmic form of Tdr kinase; and of 8.2 μ M, 7.0 μ M, 145 μ M and 315 μ M for the mitochondrial form of the enzyme from human leukaemic blast cells.

Deoxycytidine and the deoxypurines (deoxyadenosine and deoxyguanosine) do not act as phosphate acceptors for Tdr kinase from Yoshida sarcoma tissue (Hashimoto *et al.*, 1972). On the other hand Lee and Cheng (1976c) have shown that deoxycytidine may be phosphorylated by the mitochondrial form of Tdr kinase from human leukaemic blast cells.

Phosphate Donors

In general ATP and dATP have been shown to be the most effective phosphate donors for Tdr kinases from calf thymus (Her and Momparler, 1971);

rat regenerating liver (Bresnick *et al.*, 1970; Walboomers and Wanka, 1974); human adult and foetal liver (Taylor *et al.*, 1972); and HeLa cells (Taylor *et al.*, 1972; Kit and Leung, 1974).

Other purine and pyrimidine nucleoside or deoxynucleoside triphosphates may in some cases also function as phosphate donors, although to a lesser degree than ATP or dATP. Yoshida sarcoma Tdr kinase can utilise CTP and GTP (Fujii *et al.*, 1972), while calf thymus Tdr kinase can use GTP, UTP, CTP, dGTP and dCTP (Her and Momparler, 1971). Studies on Tdr kinases from human adult and foetal liver have shown that the foetal liver and human HeLa and KB cell-line forms of the enzyme are specific for ATP or dATP, whereas human adult liver Tdr kinase can, in addition, use CTP, UTP or GTP (Taylor *et al.*, 1972). The cytoplasmic form of Tdr kinase from human leukaemic blast cells similarly shows a specificity for ATP and dATP, whereas the mitochondrial form of the enzyme from leukaemic blast cells can also use CTP as a phosphate donor (Lee and Cheng, 1976c).

dTTP serves as a feed-back inhibitor of Tdr kinase, and is not effective as a phosphate donor in reactions catalysed by this enzyme.

Inhibitors

Thymidine kinase from all tissue and cell sources studied has been shown to be subject to end-product inhibition by dTTP. The enzyme form from rat foetal liver is more susceptible to inhibition by dTTP than that from adult liver (Klemperer and Haynes, 1968). On the other hand it has been observed that the Tdr kinase forms from human foetal and adult liver (Taylor *et al.*, 1972), and from human adrenal gland and adrenocortical carcinoma (Nawata *et al.*, 1976) are inhibited by dTTP to approximately the same extent.

The inhibition by dTTP has been shown to be competitive with

respect to thymidine for Tdr kinases from calf thymus (Her and Momparler, 1971), rat regenerating liver (Breitman, 1963), and human leukaemic blast cells (Lee and Cheng, 1976c). In the latter case K_i values for the mitochondrial and cytoplasmic forms of the enzyme of $5.7\mu\text{M}$ and $0.6\mu\text{M}$ respectively, were found.

The inhibition by dTTP with respect to the phosphate donor (ATP) has been shown to be complex for calf thymus Tdr kinase (Her and Momparler, 1971). At low ATP levels the inhibition is non-competitive, but this becomes competitive at high concentrations of ATP. In the case of Tdr kinases from rat regenerating liver (Kizer and Holman, 1974), Walker carcinosarcoma (Bresnick and Thompson, 1965), and *E. coli* (Okazaki and Kornberg, 1964), dTTP inhibition with respect to ATP is non-competitive at high levels of thymidine, but interactions between ATP and dTTP have been found to be complex at low thymidine concentrations.

Tdr kinase from Walker carcinosarcoma tissue in rats has been shown to exist as aggregated and disaggregated forms at, respectively, low and high ionic strengths (Bresnick *et al.*, 1966). The inhibition with respect to thymidine by dTTP for the aggregated and disaggregated forms of the enzyme was found to be, respectively, non-competitive and competitive.

dTDP and dTMP have also been shown to act as weak inhibitors of Tdr kinases from rat regenerating liver (Breitman, 1963), calf thymus (Her and Momparler, 1971), human leukaemic blast cells (Lee and Cheng, 1976c), and human adult and foetal livers (Taylor *et al.*, 1972).

Deoxycytidine nucleotides have variable effects on different Tdr kinase preparations: Neither dCDP nor dCTP inhibit Tdr kinases from rat regenerating liver (Bresnick *et al.*, 1970; Breitman, 1963). dCTP has no inhibitory effect on Tdr kinases from foetal, neonatal or adult rat

cardiac muscle (Gillette and Claycomb, 1974), and shows very little inhibitory effect on the HeLa cell forms of the enzyme which are specific for ATP and dATP as a phosphate donor (Kit and Leung, 1974). The less specific forms of the enzyme from the HeLa cells (Kit and Leung, 1974) are sensitive to dCTP inhibition.

The cytoplasmic Tdr kinase form from human leukaemic blast cells is less sensitive to dCTP inhibition than the mitochondrial form of the enzyme (Lee and Cheng, 1976c). dCTP inhibits Tdr kinase from human adult liver, but has no effect on the forms of the enzyme from foetal liver or human HeLa or KB cells (Taylor *et al.*, 1972). Similarly the Tdr kinase preparation from human adrenal gland is susceptible to dCTP inhibition, whereas the Peak I preparations from hyperplastic and cancerous human adrenal glands are not affected by this nucleotide (Nawata *et al.*, 1976). These findings are in agreement with the suggestion by Roux (1973) that the sensitivity of Tdr kinase to dCTP inhibition appears to correlate inversely with the cellular growth rate.

Regulation of Thymidine Kinase Activity

Increased Tdr kinase activity in cultured Chang liver cells, in which the *de novo* pathway of dTTP synthesis has been blocked using amethopterin, has been shown to require *de novo* synthesis of the enzyme, since puromycin or Actinomycin D prevents the expected increase in Tdr kinase activity (Eker, 1968). When Actinomycin D was administered 5 hours after amethopterin, only a slight inhibition of the increase in Tdr kinase activity was observed, which suggests that DNA-directed synthesis of RNA is necessary for increased Tdr kinase activity.

The rate of synthesis of Tdr kinase increases some 10-fold during periods of increased cell-division (Bello, 1974), and this increase in Tdr kinase activity during cell-proliferation may involve a derepression

of the gene responsible for Tdr kinase synthesis by a thymidine phosphorylated derivative, possibly dTTP (Eker, 1968).

Tdr kinase appears to be inducible, since studies on levels of activity of the enzyme from the livers of 10 day old developing mice showed that premature termination of nursing led to a decrease of up to 56% of the soluble form of the enzyme within 6 hours (Adelstein *et al.*, 1971). Glucocorticoids have been shown to induce Tdr kinase activity in perfused livers or spleens of rats bearing solid Yoshida sarcoma tumours (Sakata, 1975).

Cyclic-AMP has no effect on the activity of the cytoplasmic and mitochondrial forms of Tdr kinase from human leukaemic blast cells (Lee and Cheng, 1976b), or on the *in vitro* activity of the enzyme from rat cardiac muscle (Gillette and Claycomb, 1974). Activities of the Peak I and Peak II forms of Tdr kinase from ascites sarcoma cells have been shown to be affected differently by the presence of c-AMP (Hamazaki, 1973); the activity of the Peak I fraction decreases, whereas that of the tumour-associated Peak II fraction increases.

AIMS OF THE PRESENT STUDY

The relatively unique supply of human hepatoma tissue available in Southern Africa prompted an investigation of the levels of activity of the key enzymes of DNA synthesis in this tissue, as an extension of work already carried out by others in this laboratory on the levels of enzymes of carbohydrate metabolism (Balinsky *et al.*, 1973a,b,c; Cayanis and Balinsky, 1975). Biochemical studies on pyrimidine and deoxyribonucleic acid synthesis in human primary liver cancer have been restricted to the very recent measurement of the incorporation of ^3H -thymidine into DNA (Weber, 1976b).

Since thymidine is unique to DNA, being replaced by uridine in RNA, the levels of three key enzymes of thymidine metabolism, namely thymidine kinase, thymidylate kinase, and thymidylate synthetase; in addition to DNA polymerase and deoxycytidylate deaminase, were measured in human hepatoma tissue and compared with the levels in the corresponding host liver tissue and in human normal liver. Foetal liver, unlike adult liver, represents growing liver tissue, hence the levels of the above enzymes were also studied in this tissue. Two cell-lines derived from human hepatomas were available and were also assayed for comparative purposes. Oesophageal cancer is prevalent in South Africa and samples of the tissue and a derived cell-line were assayed as available.

In order to test the assay methods used, and to confirm that assay results were comparable with published values for rat hepatomas, levels of these enzymes in a 3'Me-DAB chemically-induced rat transplantable hepatoma were assayed.

Thymidine kinase has been shown to play an important role in DNA synthesis in rat foetal, regenerating, and cancerous liver. Different

forms of the enzyme have been described for human adult liver, and for human foetal liver or HeLa and KB cell-lines, and an additional thymidine kinase isozyme has been described in rat transplantable hepatomas. Furthermore it has been shown that the isozymes of thymidine kinase in these human or rat tissues differ with respect to their physical and kinetic properties. It was therefore decided to purify the thymidine kinase forms from human normal liver and from hepatoma, and to compare some of their physical and kinetic properties.

CHAPTER 2

LEVELS OF SOME KEY ENZYMES OF DNA SYNTHESIS IN RAT AND HUMAN NORMAL ADULT, HOST, CANCEROUS AND FOETAL TISSUES, AND CELL-LINES

	<u>Page</u>
<u>MATERIALS AND METHODS</u> -----	59
<u>Materials</u> -----	59
Chemicals, Reagents and Equipment -----	59
Substrates and Cofactors -----	59
Radiochemicals -----	59
Reagents for Scintillation Counting -----	60
Other Chemicals and Reagents Used -----	60
Equipment and other Materials -----	60
Tissues and Cells -----	61
Human Hepatoma and Host Liver -----	61
Human Normal Liver -----	62
Human Foetuses -----	62
Human Normal and Cancerous Oesophageal Tissue -----	62
Human Meningioma -----	62
Rats and Rat Hepatomas -----	63
Rat Hepatoma Cell-lines -----	63
Human Hepatoma Cell-lines -----	64
Human Oesophageal Cancer Cell-line -----	64
<u>Methods</u> -----	64
Preparation of Enzyme Extracts -----	64
Tissues -----	64
Cells -----	65
Assays -----	65
DNA Polymerase -----	66
Thymidine Kinase -----	68
Thymidylate Kinase -----	69
Deoxycytidylate Deaminase -----	71
Spectrophotometric Assay for dCMP Deaminase -----	73
Thymidylate Synthetase -----	73
Estimation of Radioactivity -----	75

	<u>Page</u>
Protein Determination -----	76
Statistical Evaluation of Results -----	76
<u>RESULTS</u> -----	77
Comparison of Isotopic and Spectrophotometric Assays for dCMP Deaminase -----	77
Comparative Studies of Levels of Some Enzymes of DNA Synthesis -----	77
Rat Normal and Host Livers, Transplantable Hepatomas, and Cell-lines Derived from the Rat Transplantable Hepatoma -----	77
Stability of the Enzymes Studied -----	77
Enzyme Levels -----	79
Human Adult Normal and Foetal Livers, Hepatomas, and Cell-lines Derived from Human Hepatomas -----	90
Stability of the Enzymes Studied -----	90
Enzyme Levels in Liver Tissues -----	90
Levels of Enzymes in Foetal Liver at Different Gestational Ages -----	93
Enzyme Levels in Human Hepatoma Cell-lines -----	93
Human Foetal, Host and Cancerous Oesophageal Tissue, and an Oesophageal Cancer Cell-line -----	96
Human Meningioma and Human Foetal Brain -----	98
<u>DISCUSSION</u> -----	101
Assay Methods -----	101
Stability of the Enzymes Assayed from Human Tissues --	102
Studies of Levels of Some Enzymes of DNA Synthesis in Human and Rat Tissues and Cell-lines -----	103
Rat Normal and Host Livers, Hepatomas, and Hepatoma Cell-lines -----	103
Human Normal and Host Livers, and Hepatomas -----	107
Human Foetal Livers -----	110
Human Hepatoma Cell-lines -----	112
Human Foetal, Host and Cancerous Oesophageal Tissue, and an Oesophageal Carcinoma Cell-line --	113
Human Foetal Brain and Meningiomas -----	113
The Role of Deoxycytidylate Deaminase in dTTP Synthesis -----	114
<u>CONCLUSIONS</u> -----	114
<u>SUMMARY</u> -----	115

CHAPTER 2

LEVELS OF SOME KEY ENZYMES OF DNA SYNTHESIS IN RAT
AND HUMAN NORMAL ADULT, HOST, CANCEROUS AND FOETAL
TISSUES, AND CELL-LINESMATERIALS AND METHODSMATERIALSChemicals, Reagents and EquipmentSubstrates and Cofactors

Crystalline deoxythymidine (Tdr); disodium deoxythymidine 5'-monophosphate (dTMP); sodium deoxythymidine 5'-diphosphate (dTDP); sodium deoxythymidine 5'-triphosphate (dTTP); sodium 2'-deoxyuridine 5'-monophosphate (dUMP); sodium 2'-deoxycytidine 5'-monophosphate (dCMP); sodium 2'-deoxycytidine 5'-triphosphate (dCTP); sodium 2'-deoxyguanosine 5'-triphosphate (dGTP); disodium 2'-deoxyadenosine 5'-triphosphate (dATP); deoxyribonucleic acid (DNA), type V, sodium salt; d,l-L-tetrahydrofolic acid (THFA), grade III were obtained from the Sigma Chemical Company, St Louis, Missouri, United States of America. Disodium adenosine 5'-triphosphate (ATP) was obtained from Boehringer Mannheim (SA) (Pty) Ltd., Bryanston, Johannesburg, South Africa. 5-Methyl-deoxycytidylic acid (5-Me-dCMP) was purchased from Calbiochem A.G., Lucerne, Switzerland.

Radiochemicals

Deoxythymidine-(methyl-³H) 5'-triphosphate, ammonium salt, 10Ci/mmol, (³H-dTTP); deoxythymidine-(methyl-³H), 5Ci/mmol, (³H-Tdr); deoxythymidine-(methyl-³H) 5'-monophosphate, ammonium salt, 20Ci/mmol, (³H-dTMP); deoxycytidine-5-³H 5'-monophosphate, ammonium salt, 13.4Ci/mmol, (³H-dCMP); and deoxyuridine-5-³H 5'-monophosphate, ammonium salt, 5Ci/mmol, (³H-dUMP) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. These radiochemicals were diluted with deionised water or unlabelled nucleotide solution on arrival, and were stored at -20°C, with

the exception of ^3H -Tdr which was stored at 4°C in accordance with the manufacturer's instructions.

Reagents for Scintillation Counting

2,5-Diphenyloxazole (PPO), scintillation grade; 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), scintillation grade; p-bis-(σ -methylstyryl)-benzene (bis-MSB), scintillation grade; and Instagel were obtained from the Packard Instrument Company, Inc., Downers Grove, Illinois, United States of America.

Other Chemicals and Reagents Used

Tris-(hydroxymethyl)-aminomethane (Tris); toluene and formaldehyde (both of guaranteed analytical grade), and Folin-Ciocalteu's Phenol Reagent were obtained from E. Merck, Darmstadt, Germany. Norit A (activated charcoal) was purchased from the Sigma Chemical Company, St Louis, Missouri, United States of America. Crystallised bovine plasma albumin was obtained from the Armour Pharmaceutical Company Ltd., Eastbourne, England. 2-Mercaptoethanol and Folin Ciocalteu's Phenol Reagent were purchased from BDH Biochemicals Ltd., Poole, England.

All other reagents and chemicals used were of analytical grade and were obtained from: Hopkin and Williams, Chadwell Heath, Essex, England; British Drug Houses, BDH Laboratory Chemicals Division, Poole, England; E. Merck, Darmstadt, Germany; Mallinkrodt, Inc., St Louis, Missouri, United States of America.

Equipment and Other Materials

Swinnex filter units and MF-Millipore filters (13mm, 0.22μ) were purchased from the Millipore Corporation, Bedford, Massachusetts, United States of America. Whatman DE 81 DEAE Cellulose ion-exchange chromatography paper and Whatman GF/C glass fibre discs (2.1cm diameter) were obtained from W. & R. Balston Ltd., England.

Tissues and Cells

Human Hepatoma and Host Liver (apparently normal tissue from a site adjacent to the tumour) were obtained at autopsy. Patients were male Blacks, usually of the Shangaan tribe. Some patients had received chemotherapy in the form of adriamycin, 5-fluorouracil, methotrexate or urea, although often less than three doses of a drug were administered prior to death and many patients presented at such an advanced stage that no therapy against the tumour was attempted. Autopsies were performed as soon as possible and tissues were obtained between two and eight hours after death. The tissues were provided by Dr E.W. Geddes, and autopsies were performed by pathologists of the National Research Institute for Occupational Diseases. All tissues were washed well to remove bile, and samples were submitted to the Histopathology Department of The South African Institute for Medical Research for confirmation that necrosis was less than 15%. Samples showing greater than 15% necrosis were not assayed. Assays were performed as soon as possible after receiving the tissue. In a few instances tumorous and host liver were stored frozen at -20°C .

On one occasion a sample of hepatoma with a small amount of 'host' liver was obtained at a hemi-hepatectomy performed by Mr B. Rabinowitz of Baragwanath Hospital. The patient was a middle aged Black female with a primary carcinoma of the liver. The tissues were placed on ice immediately after removal and assayed within three hours. Some tissue was stored at -20°C or -180°C for enzyme stability studies.

A single sample of biopsied human hepatoma was obtained during an exploratory laparotomy. The patient was a young Shangaan male. Assays were performed within three hours of surgery.

Samples of human normal liver were obtained at autopsy from organ donors with the co-operation of Dr G.M.G. Maier of the Tissue Bank of The South African Institute for Medical Research. In all cases it was confirmed that there was no liver pathology. Enzyme assays were performed immediately.

Human Foetuses were obtained from the Johannesburg General Hospital, with the permission of Professor L. van Dongen. Foetuses were obtained following either spontaneous or, in some cases, therapeutic abortions. Spontaneously aborted foetuses were rejected if they did not appear to have been viable immediately prior to abortion. The gestational ages of the foetuses were estimated by total weight and crown-rump measurements (Documenta Geigy, Scientific Tables, Seventh Edition, 1976, page 691), and were found to range from nine weeks to twenty eight weeks. Where possible livers and, in some cases, brain or oesophageal tissues were removed on obtaining the foetus, usually within two hours. The gall bladder was separated from the liver by dissection. Enzyme assays on these human foetal tissues were performed immediately.

Human Normal and Cancerous Oesophageal Tissue was obtained at surgical removal of a cancerous section of the oesophagus, or by biopsy during oesophagoscopy. These tissues were made available by Mr J. Hunt of Baragwanath Hospital. Tissue samples were stored on ice during transport. Enzyme assays were performed within three hours of biopsy or surgery.

Human Meningioma tissue was obtained at surgery from Mr K. Lewer-Allen. Tissues were stored on ice and assayed within three hours of removal.

Rats used were of the Wistar strain. Rat Hepatomas had been induced by including the hepatocarcinogen 3'-methyl-4-dimethylamino-azobenzene (3'Me-DAB) in the rat diet (Albrecht *et al.*, 1972). These hepatomas were subsequently serially transplanted into the hind legs of rats. Tumours normally required about two weeks to attain a suitable size, with considerable necrosis developing beyond this period. Rats with implanted tumours were maintained on a standard laboratory diet and were fed *ad libitum*. The livers of rats bearing implanted 3'Me-DAB tumours were termed 'host' livers. Rats were sacrificed by cervical dislocation, between 09h00 and 11h00. This time schedule was strictly adhered to, since it has been shown that levels of Tdr kinase are subject to diurnal variation (Potter *et al.*, 1966; Barbiroli *et al.*, 1972). Hepatomas and host livers were assayed within four hours of removal. Tissues were also stored at -20°C for varying lengths of time to determine the stability of the enzymes assayed. Rats of the same strain were used in the studies on enzyme levels in normal liver.

Rat Hepatoma Cell-lines were supplied by Dr L.D. Nourse and Professor O.W. Prozesky. These cell-lines were designated NRH and PRH respectively. Both cell-lines were developed from the 3'Me-DAB-induced transplantable hepatoma of Dr C.F. Albrecht. The NRH cell-line was grown at 37°C in Eagle's Minimum Essential Medium containing Hank's base and L-glutamine, but without sodium bicarbonate. This medium was supplemented with 0.26M ascorbic acid; 0.1mM glycine; 0.1mM serine; 9mM NaHCO_3 ; penicillin (50units/ml); streptomycin (50units/ml) and 5% foetal bovine serum albumin (Albrecht *et al.*, 1975). The PRH cells were grown at 36°C with 5% CO_2 in a 50/50 mixture of Eagle's Basal Medium and Dulbecco's Modified Eagle's Medium, supplemented with penicillin G (100units/ml);

streptomycin (0.1mg/ml) and 10% foetal calf serum (Prozesky *et al.*, 1973). The culture medium used was one chosen for optimal growth conditions of a human hepatoma cell-line, with the result that growth conditions for these PRH cells were found to be sub-optimal. On the other hand the medium in which the NRH cells were cultured was chosen for optimal growth of this cell-line. In both cases cells were harvested by trypsinisation followed by washing in serum free medium.

Human Hepatoma Cell-lines were developed and supplied by Professor O.W. Prozesky and Dr J. Alexander. These cell-lines were designated PHH and AHH respectively. The PHH cells were grown and harvested as described above for the PRH cell-line. These cells were found to be very rapidly growing, in contrast to the AHH cell-line which was characterised by very slow growth.

The AHH cells and a Human Oesophageal Cancer Cell-line, also provided by Dr J. Alexander and designated AHO, were grown at 37°C in Eagle's Minimum Essential Medium containing essential amino acids; penicillin (100µg/ml) and streptomycin (100µg/ml); and supplemented with 10% foetal bovine serum (containing 200µg/ml each of penicillin and streptomycin, and 100µg/ml of Mycostatin). Cells were harvested by trypsinisation followed by washing with serum-free medium.

METHODS

All procedures were carried out at 4°C unless otherwise stated. All solutions were prepared using deionised water.

Preparation of Enzyme Extracts

Tissues

The same buffer was used to extract all of the enzymes assayed except dCMP deaminase. The homogenising buffer contained 50mM Tris-HCl (pH 7.5), 250mM sucrose, 5mM β-mercaptoethanol and 1mM EDTA. In

the case of dCMP deaminase preparations the homogenising buffer contained, in addition to the above, 0.5mM dCTP which stabilises this enzyme. Mercaptoethanol and, in the case of dCMP deaminase, dCTP were added to the stock buffer immediately prior to homogenisation. Homogenising buffer was used to prepare 20% (weight for volume) homogenates of the normal or cancerous tissues. Homogenisation was performed using five strokes in a Potter-Elvehjem homogeniser with a moderately tight-fitting ground-glass pestle. Oesophageal cancer and meningioma tissues were usually rather fibrous and therefore required additional homogenisation to effect adequate cellular disruption.

Homogenates were centrifuged at 38 000 x g for sixty minutes at 4°C, using a 16 x 15ml rotor in an MSE 18 refrigerated centrifuge. As the enzymes to be assayed were located in the post-microsomal or soluble fractions, the clear supernatant of each homogenate was retained for assay.

Cells.

Harvested cells in serum-free medium were washed three times in 0.9% sodium chloride. The cell pellet was then suspended in five to ten volumes of the same homogenising buffer as was used for the tissues. Cells were disrupted by three cycles of freezing and thawing using a dry-ice acetone bath. The extracts were centrifuged at 38 000 x g for sixty minutes at 4°C, and the supernatants were retained for enzyme assays.

Assays

All assays used involved the conversion of radioactively-labelled substrate into radioactively-labelled products. Each assay was checked to ensure that the reaction rate was linear over the time period assayed and that not more than 10-20% of the labelled substrate was converted to product during the assay period. All assays were performed in duplicate.

DNA Polymerase

Principle of the assay

The method used was based on those of Mantsavinos (1964), Shepherd and Keir (1966) and Burr-Furlong (1967). The principle involved is that ^3H -dTTP incorporated into newly-synthesised DNA may be measured by precipitating the DNA onto a low quenching inert support such as a glass fibre disc. Unreacted ^3H -dTTP is then washed off and the ^3H -dTTP label incorporated into DNA and precipitated on the disc is counted using a suitable scintillant.

Determination of optimal conditions

The method of Shepherd and Keir (1966) was used except that the concentration of unlabelled dTTP was decreased to increase the specific activity of ^3H -dTTP. This was necessary because the activity of the enzyme was found to be very low in human or rat normal adult livers.

The activity of the enzyme was assayed using both heat-denatured and native template DNA's extracted from calf thymus. Denatured DNA was prepared by heating a 2mg/ml native DNA preparation in a boiling water bath for five minutes and then cooling the preparation rapidly on ice. Two types of calf thymus DNA were commercially available, that from Boehringer Mannheim being highly polymerised with a molecular weight ten-fold greater than that supplied by the Sigma Chemical Company. It was found that the highly-polymerised Boehringer preparation was less effective as a DNA template whether in native or denatured form, and it was therefore decided to use the Sigma type V DNA from calf thymus as template DNA.

The pH optimum of the enzyme from human foetal liver was found to be 7.5 which is similar to the results of Shepherd and Keir (1966), who found an optimum pH of 7.2-7.4 for the enzyme partially purified from Landschütz Ascites-tumour cells.

Final assay conditions used

Assay mixtures contained, in a total volume of 250 μ l, 20mM Tris-HCl buffer (pH 7.5); 0.4mM EDTA; 50mM KCl; 8mM MgSO₄; 5mM β -mercaptoethanol; 90 μ M ³H-dTTP (approximately 20 μ Ci/ μ mole of dTTP); 200 μ M dATP; 200 μ M dGTP; 200 μ M dCTP; 50 μ g of native or denatured DNA; and up to 1mg protein. The reaction was started by the addition of the four deoxyribonucleoside triphosphates. A 100 μ l aliquot was withdrawn immediately as a zero-time sample. The remaining 150 μ l of assay mixture was incubated for 20 minutes at 37°C. Reactions were terminated by rapidly freezing assay mixtures in an acetone dry-ice bath. After thawing, bovine serum albumin (10 μ l per 50 μ l assay mixture) was added to the zero and twenty minute assay tubes to assist in the precipitation of DNA onto the glass fibre discs. A 50 μ l aliquot of each assay mixture was spotted onto a numbered Whatman GF/C glass-fibre disc which had previously been soaked in sodium pyrophosphate (100mM) and allowed to dry. The presence of pyrophosphate prevented any further polymerising activity.

Discs were dried briefly in air and then dropped into a beaker containing ice-cold 5% TCA. The discs were washed twice on a Buchner funnel using 5% TCA, and dried by washing with absolute ethanol and with anaesthetic ether. Incorporated radioactivity was counted by placing each disc in a scintillation vial containing 10ml of scintillation cocktail and counting as described on page 75. A 'total counts control' was prepared by spotting a 50 μ l aliquot of the twenty minute reaction mixture onto a glass fibre disc, which was dried at 60°C and counted.

Activities are expressed as mUnits per gram of protein, one Unit being defined as the incorporation of 1 μ mole ³H-dTTP into DNA per minute at 37°C.

Using the assay procedure described DNA polymerase activities from rapidly dividing human foetal liver or rat hepatoma tissues were found to be linear with respect to time for up to 45 minutes at protein concentrations of up to 1mg per assay. Specific activities of between 0.5 and 250Units per gram of protein could be measured.

Thymidine Kinase (Tdr kinase)

Principle of the assay

Tdr kinase catalyses the phosphorylation of Tdr to dTMP by ATP. The assay method used was that of Burr-Furlong (1963) as modified by Elford *et al.* (1970). The principle involved is that, since only nucleotides or deoxynucleotides (in this case dTMP, dTDP, and dTTP) bind to Whatman DE 81 ion-exchange chromatography paper, unconverted ^3H -Tdr may be removed by washing, and the disc may then be counted to determine the amount of incorporated radioactivity.

Determination of optimal assay conditions

The methods generally used for the assay of Tdr kinase include either α -glycerophosphate (Okuda *et al.*, 1972) or 3-phosphoglyceric acid (Sneider *et al.*, 1969; Salser and Balis, 1973) in the assay mixture to decrease hydrolysis of ATP due to contaminating phosphatases. However in the present study it was found that rat hepatoma Tdr kinase activity was decreased by 9% and 64% respectively in the presence of α -glycerophosphate and 3-phosphoglyceric acid. For this reason these were excluded from the Tdr kinase assay. Similar findings have been observed by Duncan and Dreosti for rat hepatoma Tdr kinase (personal communication).

Final assay conditions used

Assay mixtures contained, in a total volume of 200 μl , 50mM Tris-HCl buffer (pH 8.0); 5mM MgCl_2 ; 5mM ATP (adjusted to pH 7 with sodium bicarbonate); 50 μM ^3H -Tdr (approximately 20 μCi per μmole Tdr); and up to

2mg protein. The reaction was initiated by the addition of ^3H -Tdr. The contents of each assay tube was mixed and a zero-time aliquot of 100 μl was immediately withdrawn. The remaining assay mixture was incubated at 37°C for 20 minutes. Assays were terminated by placing the tubes in a boiling water bath for 4 minutes. The tubes containing the assay mixtures were centrifuged to spin down precipitated protein, and 25 μl aliquots of the zero-time and twenty minute assay mixture supernatants were spotted onto 2cm diameter DE 81 discs.

The discs were dried in air for about one minute and were then washed on a Buchner funnel using 1mM ammonium formate (10ml per disc) and water. This washing cycle was repeated twice. The discs were dried, placed in 10ml of scintillation cocktail, and the incorporated radioactivity was determined as described on page 75. A total count measurement for each assay was determined by spotting a 25 μl aliquot of each twenty minute assay mixture supernatant onto a DE 81 disc, which was dried and counted without washing.

Activities are expressed as mUnits per gram of protein, 1 Unit being defined as the production of 1 μmole of ^3H -dTTP per minute at 37°C.

Using extracts of rapidly dividing tissue, eg. rat hepatoma, the rate of reaction for Tdr kinase was found to be linear with respect to time for assay periods of up to 45 minutes using protein concentrations of up to 2mg per assay. Specific activities of between 1.0 and 600 mUnits per gram of protein could be measured.

Thymidylate Kinase (dTTP Kinase)

Principle of the assay

The assay method was based on those of Gordon *et al.* (1968) and Snider *et al.* (1969). The principle involved is that the substrate ^3H -dTTP, and product ^3H -dTDP (and possibly ^3H -dTTP) may be separated

by ascending chromatography on DE 81 ion-exchange chromatography paper using a formic acid-ammonium formate solvent.

Determination of optimal assay conditions

Optimum activity for dTMP kinase from human foetal liver was obtained at pH 7.5 in the presence of 7.5mM MgCl_2 and 50 μM dTMP. The enzyme was inhibited by sodium fluoride. This is in agreement with the results of Gordon *et al.* (1968).

Final assay conditions used

Assay mixtures contained, in a total volume of 200 μl , 50mM Tris-HCl buffer (pH 7.5); 7.5mM MgCl_2 ; 5mM ATP (adjusted to pH 7 with sodium bicarbonate); 50 μM ^3H -dTMP (approximately 20 $\mu\text{Ci}/\mu\text{mole}$); and up to 2mg of protein. The reaction was initiated by the addition of ^3H -dTMP. The contents of each assay tube was mixed and a 100 μl aliquot was withdrawn immediately as a zero-time sample. The remaining assay mixture was incubated at 37°C for 20 minutes. The reactions in the 0 and 20 minute incubation samples were terminated by heating for 4 minutes in a boiling water bath. Denatured protein was spun down, and 25 μl aliquots of each supernatant were spotted onto a 30 x 2cm strip of DE 81 chromatography paper and allowed to dry.

The chromatographs were developed by ascending chromatography, using a 1N formic acid - 0.1N ammonium formate solvent system, until the solvent front had risen at least 25cm from the origin. The positions of the labelled nucleotides were located by markers consisting of 10 μl each of 1mM dTMP, 1mM dTDP or 1mM dTTP. These nucleotides fluoresce under light of 254nm wavelength and spots could thus be readily located using an ultraviolet lamp. R_f values for the thymidine deoxynucleotides were dTMP-0.6 dTDP-0.2; and dTTP-0.0.

The areas corresponding to the dTMP and dTDP-dTTP nucleotide spots were cut out and counted in 10ml of scintillation cocktail as described on page 75. The counts per minute corresponding to the zero-time dTMP spot were used as the total number of counts for each assay.

Results are expressed as mUnits per gram protein, 1 Unit being defined as the production of 1 μ mole of ^3H -dTDP or dTTP per minute at 37°C.

Human foetal liver dTMP kinase activity was found to be linear with respect to time for periods of up to 45 minutes at protein concentrations of up to 1.5mg per assay. Specific activities of 1.0 to 500 mUnits per gram of protein could be measured.

Deoxycytidylate Deaminase (dCMP Deaminase)

Principle of the assay

The assay used was based on that described by Snelder *et al.* (1969) and Maley and Maley (1969). The principle involved is that the labelled product dUMP, can be separated from the labelled substrate dCMP, by means of ascending chromatography on DE 81 ion-exchange paper, using a formic acid-ammonium formate system.

Determination of optimal assay conditions

Addition of dCTP to the homogenising medium was found to be necessary to stabilise human foetal liver and hepatoma dCMP deaminases, in agreement with the finding of Snelder and Potter (1968) for the rat hepatoma enzyme. The optimum dCTP concentration required in the homogenising mixture was 0.5mM. The pH optimum of the enzyme from human foetal liver was 7.5 to 8.5, hence a buffer of pH 8.0 was used. The same assay pH was used in the assays for dCMP deaminase from chick embryos (Maley and Maley, 1969) and rat hepatomas (Snelder *et al.*, 1969).

Final assay conditions used

Assay mixtures contained, in a final volume of 250 μ l, 40mM Tris-HCl buffer (pH 8.0); 6.0mM MgCl₂; 40 μ M dCTP; 30mM NaF; 4mM ³H-dCMP (approximately 250 μ Cl/mMole); and up to 2mg protein. The reaction was initiated by the addition of ³H-dCMP. A zero-time aliquot of 100 μ l was removed immediately and the remainder of the mixture was incubated at 37°C for 20 minutes. Reactions were terminated by heating for 4 minutes in a boiling water bath. Denatured protein was spun down, and 25 μ l aliquots of each supernatant were spotted onto 30 x 2cm strips of DE 81 chromatography paper.

The chromatogram was developed by ascending chromatography, using a 1N formic acid-0.1N ammonium formate solvent system, until the solvent front had migrated at least 25cm. The positions of labelled dCMP and dUMP were located using 10 μ l spots of appropriate deoxynucleotide markers, each at 1mM concentration. Nucleotide spots were located by their fluorescence under ultraviolet light at 254nm wavelength. The R_F value for dUMP was 0.4, that for dCMP was 0.8.

The areas corresponding to dUMP and dCMP were cut out and counted in 10ml of scintillation cocktail as described on page 75. The counts per minute obtained for the dCMP spot in zero-time assays were used as the total counts for each sample.

Activities are expressed as mUnits per gram protein, one Unit being defined as the production of 1 μ mole of ³H-dUMP per minute at 37°C.

Using the assay system described, the rate of reaction for dCMP deaminase was found to be linear with respect to time for incubation periods of up to 60 minutes at protein concentrations of up to 1.5mg per assay, for a preparation from human hepatoma. Enzyme activities of between 100 mUnits and 25 000 mUnits per gram of protein could be measured.

Spectrophotometric Assay for dCMP Deaminase

Principle of the assay

The method used was that described by Maley and Maley (1969). The method monitors the decrease in absorption at 290nm or 287nm when, respectively, dCMP is deaminated to produce dUMP, or 5-methyl-dCMP is deaminated to produce dTMP.

Final assay conditions used

Each assay mixture contained, in a final volume of 0.5ml, 60mM Tris-HCl buffer (pH 8.0); 40 μ M dCTP; 6mM MgCl₂; 2mM dCMP (or 5-Me-dCMP); and up to 4mg of protein. Reaction mixtures were incubated for 10 minutes at 37°C, and reactions were terminated by the addition of 1.5ml of cold 0.6N perchloric acid. Precipitated protein was centrifuged down, and aliquots of 0.5ml were removed and diluted to 5.0ml with water. The decrease in absorbance at 290nm (or 287nm), relative to a zero-time control, was measured using a Beckman DB spectrophotometer.

Results are expressed as units per gram of protein, one Unit being defined as the conversion of 1 μ mole of dCMP to dUMP (or 1 μ mole of 5-Me-dCMP to dTMP) per minute at 37°C.

Enzyme activity for a human hepatoma preparation was linear for up to 20 minutes at protein concentrations of up to 3mg per assay.

The isotopic and spectrophotometric assay methods for both rat and human dCMP deaminases gave comparable results.

Thymidylate Synthetase (dTMP Synthetase)

Principle of the assay

The method used was based on those of Kammen (1966) and Elford *et al.* (1970). The principle involved is that, in the course of the reaction, the hydrogen atom attached to the carbon-5 position of the dUMP pyrimidine ring is released as a proton, with substitution of a

reduced methylene group at this position to form dTMP. The released proton equilibrates with the aqueous protons. If 5-³H-dUMP is used, ³H is released during the reaction and will appear in the aqueous phase of the reaction mixture. On adsorbing all organic constituents of the reaction mixture onto activated charcoal (Norit A), the residual specific radioactivity in the aqueous phase will be proportional to the amount of dUMP converted into dTMP.

Determination of optimal assay conditions

Using an enzyme preparation from human foetal liver it was found that saturating levels of dUMP were obtained at concentrations 2.5-fold lower than those described in the literature. Lower concentrations (10 μ M) of dUMP were therefore used to ensure a higher specific activity of ³H-dUMP, and thus allow more accurate determination of the very low dTMP synthetase levels in human normal and host liver, or in rat normal liver.

No dTMP synthetase activity could be measured when using the natural coenzyme N₅,N₁₀-methylene tetrahydrofolic acid. It was found necessary to add tetrahydrofolic acid (THFA) and formaldehyde, and to allow N₅, N₁₀-methylene tetrahydrofolic acid to be generated *in situ*. This may be due to the instability of N₅, N₁₀-methylene tetrahydrofolic acid. THFA was stabilised by preparing it in 2.1mM β -mercaptoethanol. In this form THFA was stable to storage at -20°C for at least 6 months.

Final Assay conditions used

The assay mixture contained, in a total volume of 200 μ l, 20mM Tris-HCl buffer (pH 7.5); 25mM MgCl₂; 15.8mM formaldehyde; 1.0mM EDTA; 300 μ M THFA; 196 μ M β -mercaptoethanol; 10 μ M 5-³H-dUMP (approximately 250 μ Ci/ μ mole dUMP); and up to 2mg of protein. Reactions were started by the addition of ³H-dUMP.

Assay mixtures for zero-time and 15 minute incubations at 30°C were prepared. Reactions were terminated by adding 1ml of deionised water to each tube, mixing, and immediately pouring the contents of each tube into a corresponding tube containing 200mg Norit A in 1ml of water. The contents was well mixed and allowed to stand for 5 minutes. The tubes were then centrifuged to spin down the Norit A, and the supernatant was filtered using a 22µ Millipore filter. One ml aliquots of filtrates were placed in scintillation vials, each containing 9ml of Instagel. The contents of each vial was mixed well, allowed to equilibrate, and the vials were counted to determine radioactivity due to tritiated water. To determine the total counts per minute ³H-dUMP was appropriately diluted, and aliquots were counted in Instagel.

Enzyme activities are expressed as mUnits per gram protein, one Unit being defined as the conversion of 1µmole ³H-dUMP per minute at 30°C (as reflected by the appearance of an equimolar quantity of tritium-labelled water).

Human foetal dTMP synthetase activity was found to be linear with time for periods of up to 20 minutes using protein concentrations of up to 1.5mg per assay. Specific activities of between 0.3 and 150 mUnits per gram protein could be measured.

Estimation of Radioactivity

For all the enzymes assayed, except dTMP synthetase, the scintillation cocktail consisted of 5g of 2,5-diphenyloxazole (PPO) and 250mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) per litre of toluene. At a later stage 250mg of p-bis-(σ-methylstyryl)-benzene (bis-MSB) per litre of toluene was used in place of the dimethyl POPOP. This change did not affect the results. Samples were equilibrated in the dark at 4°C for at least one hour before counting.

Incorporated radioactivity was determined using a Packard Tricarb 3380 liquid scintillation counter, using a 'preset' optimum gain setting for tritium. In order to ensure that quenching in the two-phase systems was constant, each sample was simultaneously counted using a 54% gain with 50-200 window setting, and the channel ratio for the counts per minute obtained using this setting and the preset tritium-setting was determined.

Protein Determination

Protein concentrations were determined by the method of Lowry *et al.* (1951). To 1ml of each sample (which had been previously diluted to give a protein concentration of 40-120µg/ml) was added 5ml of a freshly mixed reagent containing 1 part 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 part 2.7% potassium-sodium tartrate, and 48 parts of 2% sodium carbonate in 0.1N NaOH. Tubes were mixed well and allowed to stand for at least 10 minutes. 0.5ml of BDH or Merck Folin Ciocalteu's reagent, which had been diluted with two volumes of water, was added to each tube with rapid mixing. Solutions were allowed to stand for at least 30 minutes at room temperature, and their absorbances at 750nm were then read against a reagent blank, using a Beckman DB spectrophotometer.

For each batch of protein determinations a standard curve was constructed, using bovine serum albumin protein standard.

Statistical Evaluation of Results

The levels of each enzyme assayed in the same types of tissue or cells are expressed as a mean $\pm$ the standard error of the mean (SEM). When comparing corresponding enzyme activities in comparable tissues or cells, such as normal versus cancerous liver, the significance of differences between the means $\pm$ SEM for the two sets of values was calculated by means of Student's 't' test, a value of $P < 0.01$ being considered to indicate a significant difference.

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RESULTS

COMPARISON OF ISOTOPIC AND SPECTROPHOTOMETRIC ASSAYS FOR dCMP DEAMINASE

The dCMP deaminase activities obtained with different tissues, using the isotopic assay with ^3H -dCMP and the spectrophotometric assay with either dCMP or 5-methyl-dCMP as substrates, are summarised in Table 4. For rat hepatoma the isotopic and spectrophotometric methods showed similar activities with dCMP as the substrate. However levels were about three-fold higher when 5-Me-dCMP was used as the substrate. For human foetal liver comparable levels of activity were obtained with the isotopic and spectrophotometric methods for dCMP deaminase. Preparations from human foetal liver and human hepatoma showed similar activities with dCMP and 5-Me-dCMP as substrates when using the spectrophotometric assay.

These results suggest that rat hepatoma dCMP deaminase metabolises 5-Me-dCMP more rapidly than dCMP, whereas these two substrates are deaminated at comparable rates by enzyme preparations from human foetal liver and hepatoma.

COMPARATIVE STUDIES OF LEVELS OF SOME ENZYMES OF DNA SYNTHESIS

Rat Normal and Host Livers, Transplantable Hepatomas, and Cell-lines

Derived from the Rat Transplantable Hepatoma

Stability of the Enzymes Studied

Stability studies showed that storage of rat hepatoma tissue at -20°C for one week did not lead to loss of activity of thymidine kinase, deoxycytidylate deaminase, or the form of DNA polymerase which preferentially used native template DNA. On the other hand thymidylate kinase, thymidylate synthetase, and the form of DNA polymerase which preferentially used denatured template DNA, were found to be labile to storage at -20°C even when stored in 40% glycerol.

TABLE 4

DEOXYCYTIDYLATE DEAMINASE ACTIVITIES OF RAT
AND HUMAN NORMAL AND CANCEROUS LIVER

Tissues or Cells	Activity (mUnits/gram protein)		
	Isotopic Assay (substrate-dCMP)	Spectrophotometric Assay (substrate-dCMP) (substrate- 5-Me-dCMP)	
Rat hepatoma	7 400	6 500	20 500
Human foetal liver	5 200	5 500	6 500
Human hepatoma	-	22 100	18 700

Results similar to those on page 77 were also obtained when rat liver and hepatoma tissues were stored at -70°C for one week.

Enzyme Levels

The results obtained for the enzyme levels assayed in extracts of rat normal liver, rat transplantable hepatomas, and the livers of rats bearing the hepatomas (host livers) are summarised in Table 5 and Figures 3 to 9. The figures show the activities of the enzymes (expressed as mU/gram protein) for each specimen assayed. A line is drawn to join the activity in the cancerous tissue with that in the corresponding host tissue. This permits comparison of levels in an individual, thus reducing the effects of biological variation, eg. diet. The Table gives the mean activities in each tissue, with calculation of the standard errors of the means. This permits estimation of the significance of differences between the means.

In rat normal and host liver tissues, the predominant DNA polymerase form was that which preferred native template DNA. In both cases it can be seen that the ratios of DNA polymerase activities with denatured template DNA and native template DNA were less than one. DNA polymerase from rat hepatoma showed a preference for denatured template DNA, with a ratio of activities with denatured DNA to native DNA of greater than one. Rat hepatoma DNA polymerase activities with either native or denatured template DNA were significantly higher than those of the respective host livers, as well as those from normal livers.

Tdr kinase activities in rat normal liver were not significantly different from those in host rat liver. The mean activities in hepatoma were, however, about forty-fold higher than those in host rat liver.

Activities of dTMP kinase in rat normal liver were low, while

TABLE 5

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA SYNTHESIS IN RAT NORMAL
AND HOST LIVERS AND TRANSPLANTABLE 3YMA-HAB-INDUCED HEPATOMAS

Enzyme	Activity (units/gram protein) ^a		Statistical significance of differences in levels ^{**}			
	Normal Liver	Host Liver	Hepatoma	Host Liver vs Hepatoma	Normal Liver vs Hepatoma	Normal Livers vs Host Livers
<u>DNA Polymerase</u>						
with native DNA template	2.26 ± 0.58 (6)	3.17 ± 0.25 (5)	14.6 ± 1.7 (5)	P<0.0025	P<0.0005	P<0.05
with denatured DNA template	0.81 ± 0.18 (6)	1.31 ± 0.26 (5)	20.2 ± 3.2 (15)	P<0.0005	P<0.0005	P<0.10
Ratio: Activities denatured DNA native DNA	0.58 ± 0.06 (6)	0.41 ± 0.02 (5)	1.52 ± 0.09 (5)	P<0.0005	P<0.0005	N/S
<u>Thymidine Kinase</u>	1.70 ± 0.11 (6)	2.18 ± 0.42 (15)	85.1 ± 8.4 (15)	P<0.0005	P<0.0005	P<0.20
<u>Thymidylate Kinase</u>	1.42 ± 0.11 (6)	N/D (3)	151 ± 16 (3)	P<0.0005	P<0.0005	-
<u>Thymidylate Synthetase</u>	0.59 ± 0.12 (6)	0.93 ± 0.26 (12)	42.8 ± 3.2 (12)	P<0.0005	P<0.0005	P<0.15
<u>Deoxycytidylate Deaminase</u>	50 ± 10 (6)	112 ± 30 (5)	5 680 ± 450 (5)	P<0.0005	P<0.0005	P<0.10

* Results are expressed as a mean ± the standard error of the mean, with the number of samples assayed shown in parentheses.

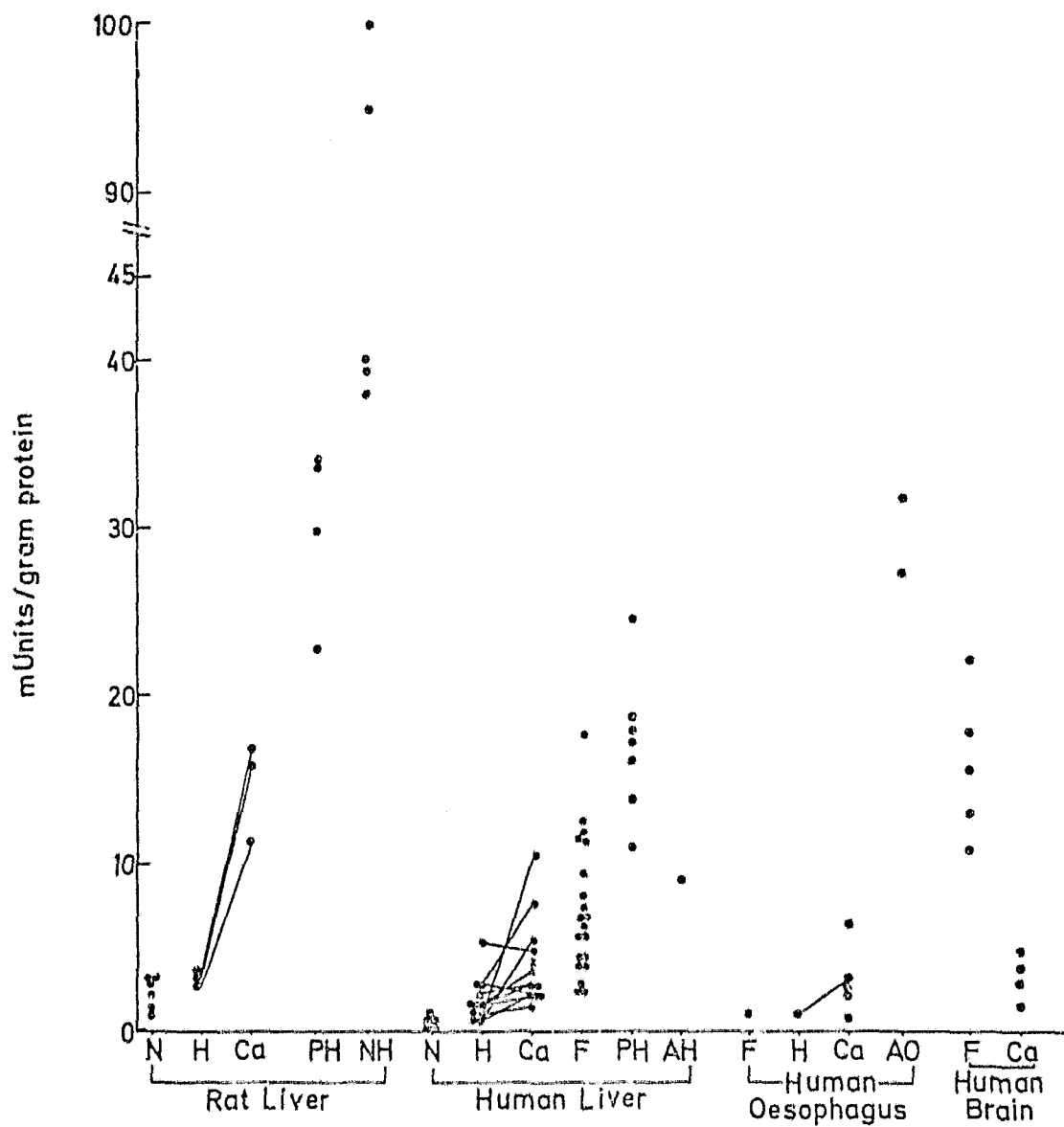
** A value of P < 0.01 is regarded as representing a significant difference.

N/D = No detectable activity.

N/S = not significantly different.

FIGURE 3

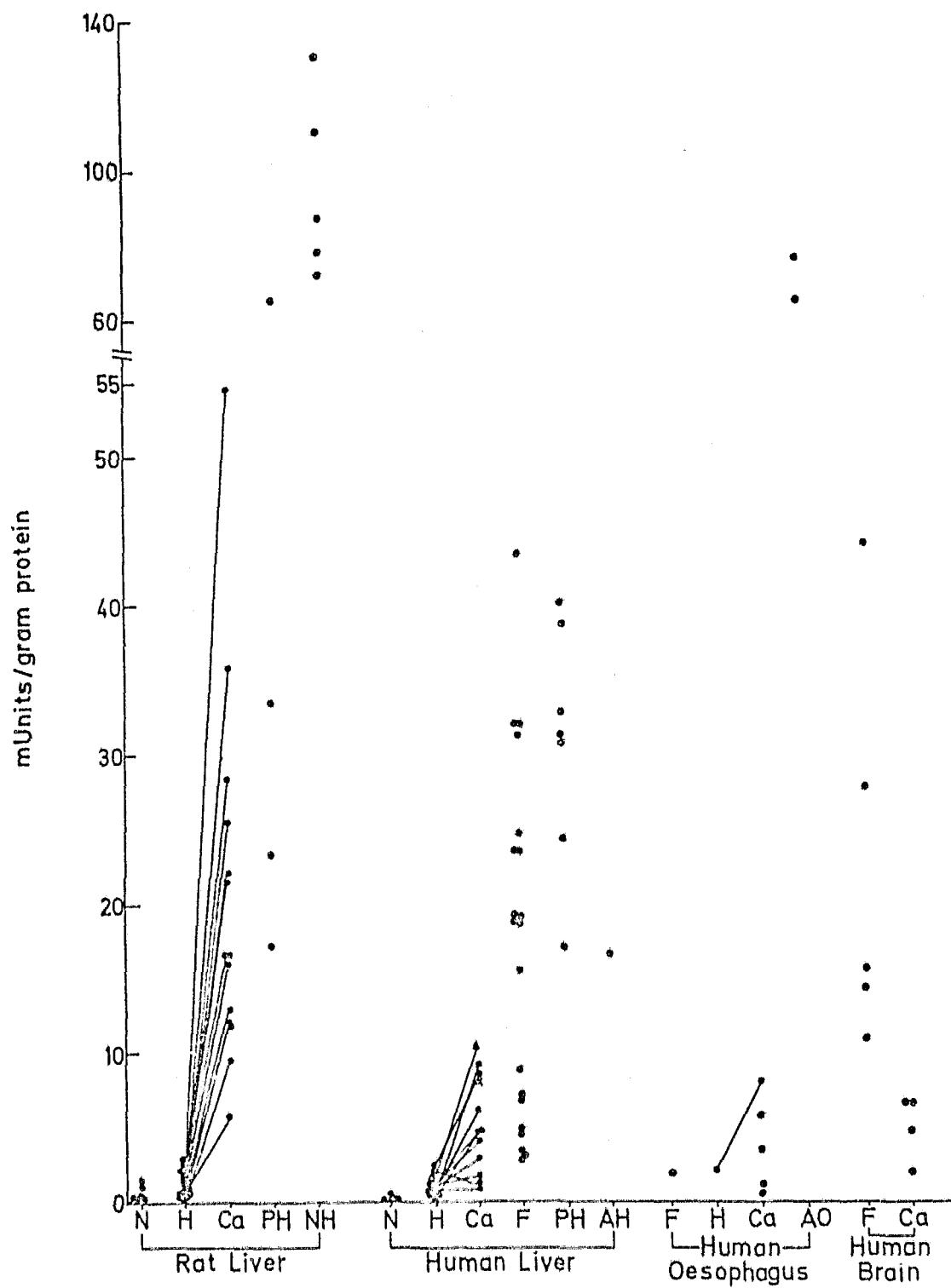
DNA POLYMERASE ACTIVITY (WITH NATIVE DNA TEMPLATE)
IN RAT AND HUMAN NORMAL ADULT, HOST, CANCEROUS AND
FOETAL TISSUES, AND CELL-LINES



A line is drawn to join the activity in cancerous tissue with that of corresponding host tissue where applicable.

Key: ● - autopsy tissue N - normal
▲ - surgically removed H - host
 human hepatoma & host F - foetal
 liver Ca - cancerous
x - surgically biopsied PH - Prozesky hepatoma cells
 human hepatoma NH - Nourse hepatoma cells
 AH - Alexander hepatoma cells
 AO - Alexander oesophageal Ca cells

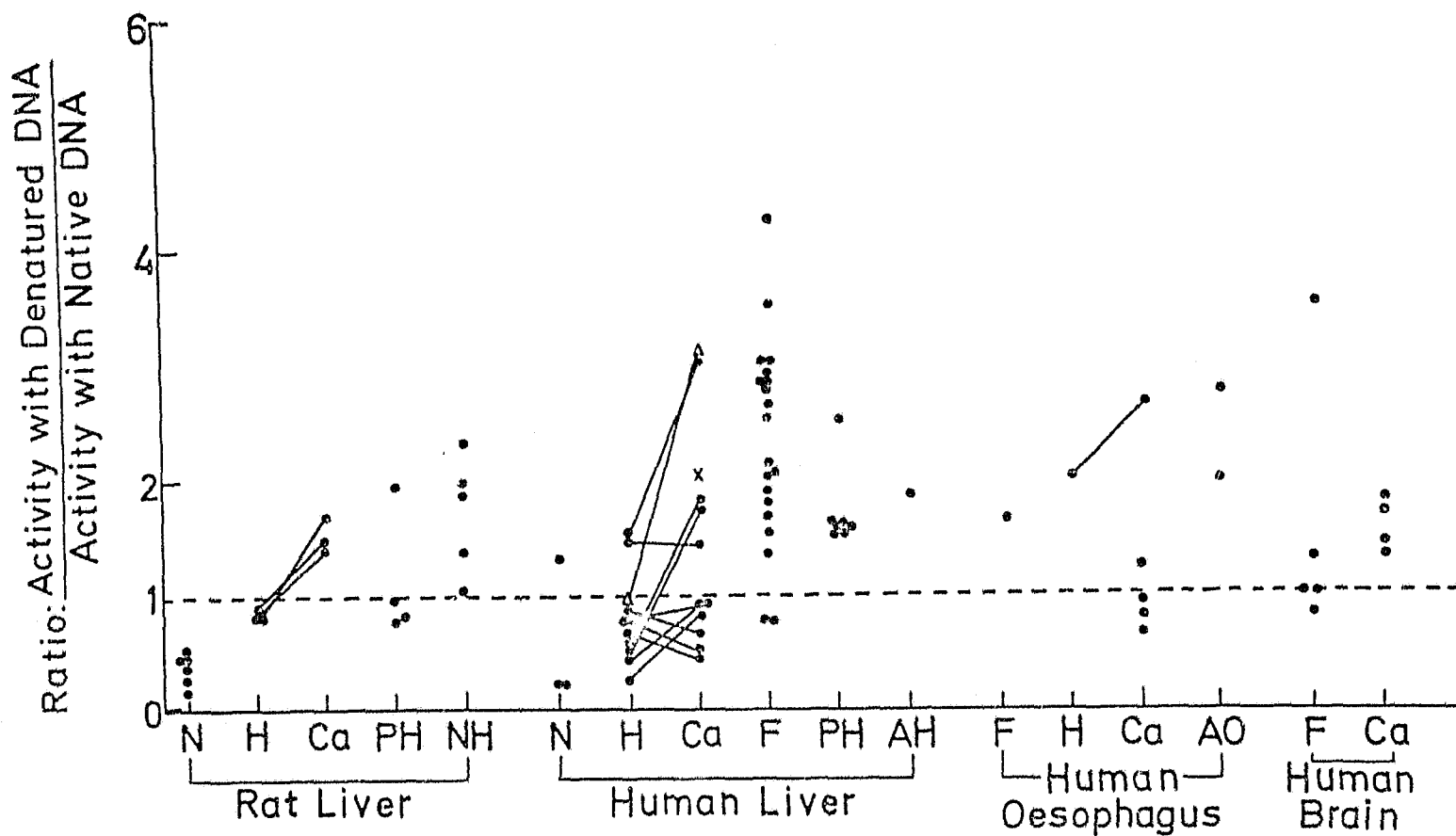
DNA POLYMERASE ACTIVITY (WITH DENATURED DNA TEMPLATE) IN RAT
AND HUMAN NORMAL ADULT, HOST, CANCEROUS AND FOETAL TISSUES,
AND CELL-LINES



Key: See Figure 3.

FIGURE 5

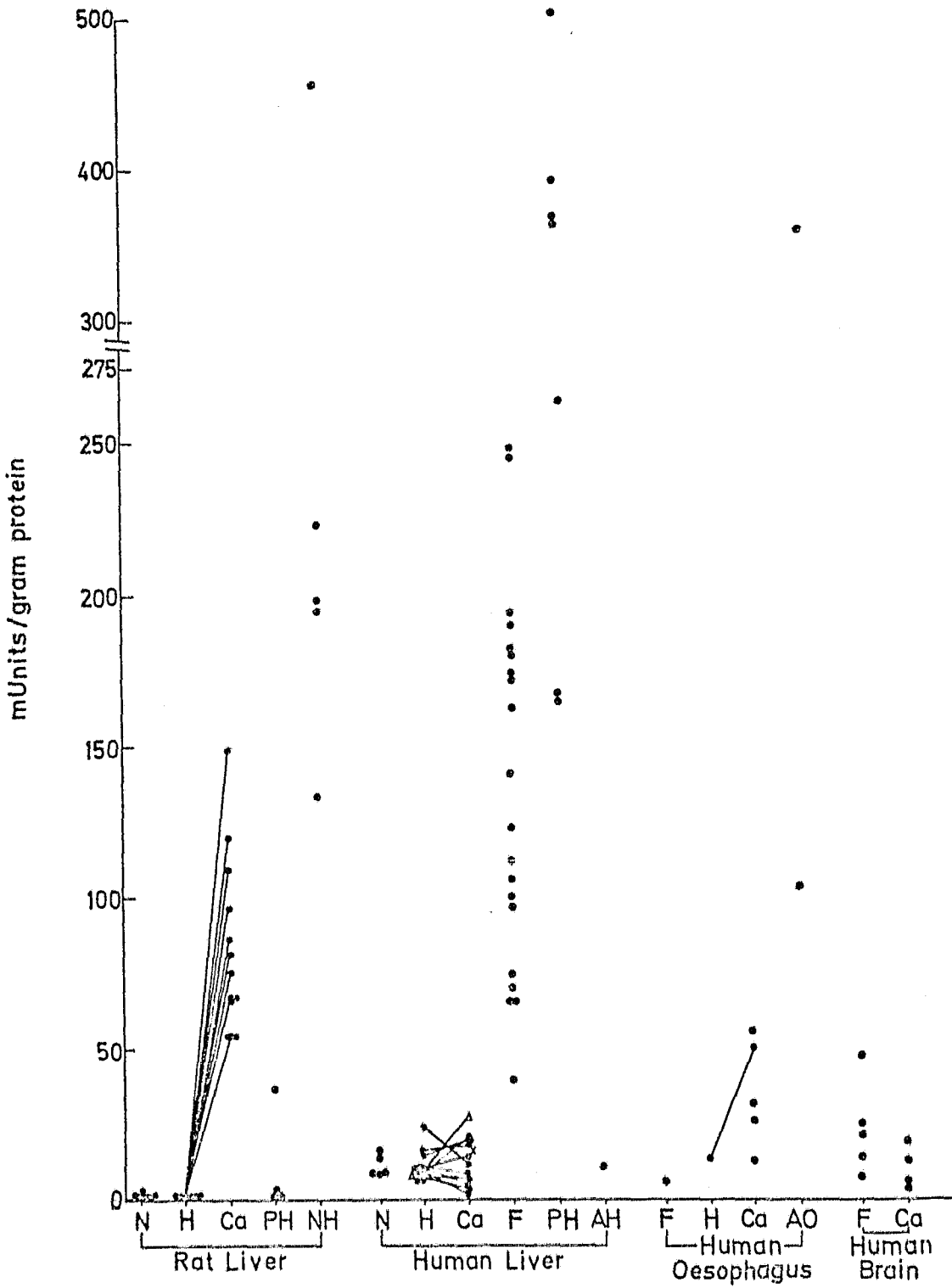
RATIO OF DNA POLYMERASE ACTIVITIES WITH NATIVE AND
DENATURED DNA TEMPLATES IN RAT AND HUMAN TISSUES, AND CELL-LINES



Key: See Figure 3.

FIGURE 6

THYMIDINE KINASE ACTIVITY IN RAT AND
HUMAN NORMAL ADULT, HOST, CANCEROUS
AND FOETAL TISSUES, AND CELL-LINES.



Key: See Figure 3.

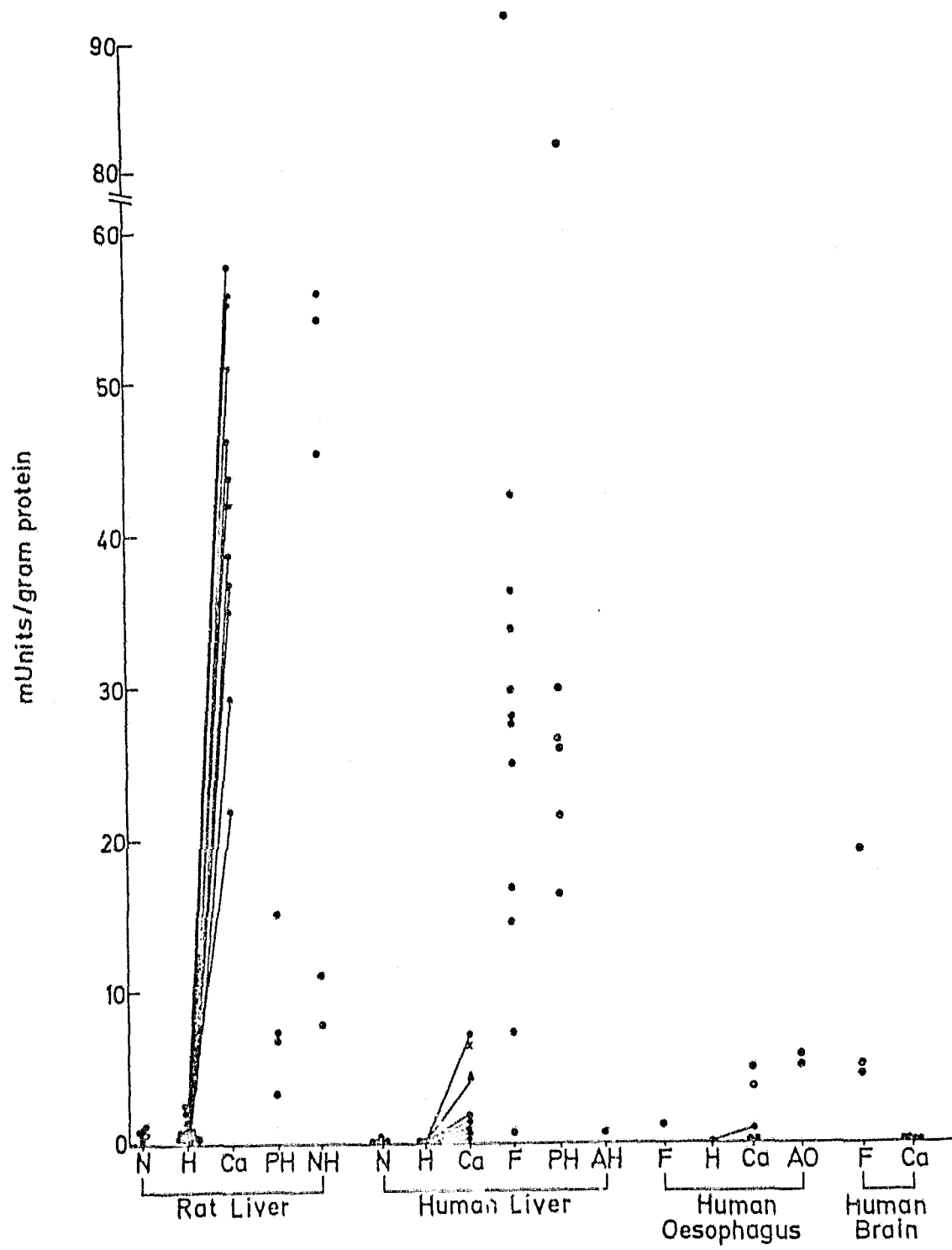
Figure 1 is a dot plot showing the distribution of mUnits/gram protein for various tissue samples. The y-axis represents mUnits/gram protein, ranging from 0 to 700. The x-axis lists the samples: Rat Liver (N, H, Ca, PH, NH), Human Liver (N, H, Ca, F, PH, AH), Human Oesophagus (F, H, Ca, AO), and Human Brain (F, Ca). Lines connect data points for the same sample type across different experiments. Rat Liver shows high values (up to 700) for PH and NH. Human Liver shows values up to 150. Human Oesophagus shows values up to 150. Human Brain shows values up to 300.

Key: See Figure 3.

FIGURE 8.

86.

THYMIDYLATE SYNTHETASE ACTIVITY IN RAT AND HUMAN NORMAL
ADULT, HOST, CANCEROUS AND FOETAL TISSUES, AND CELL-LINES

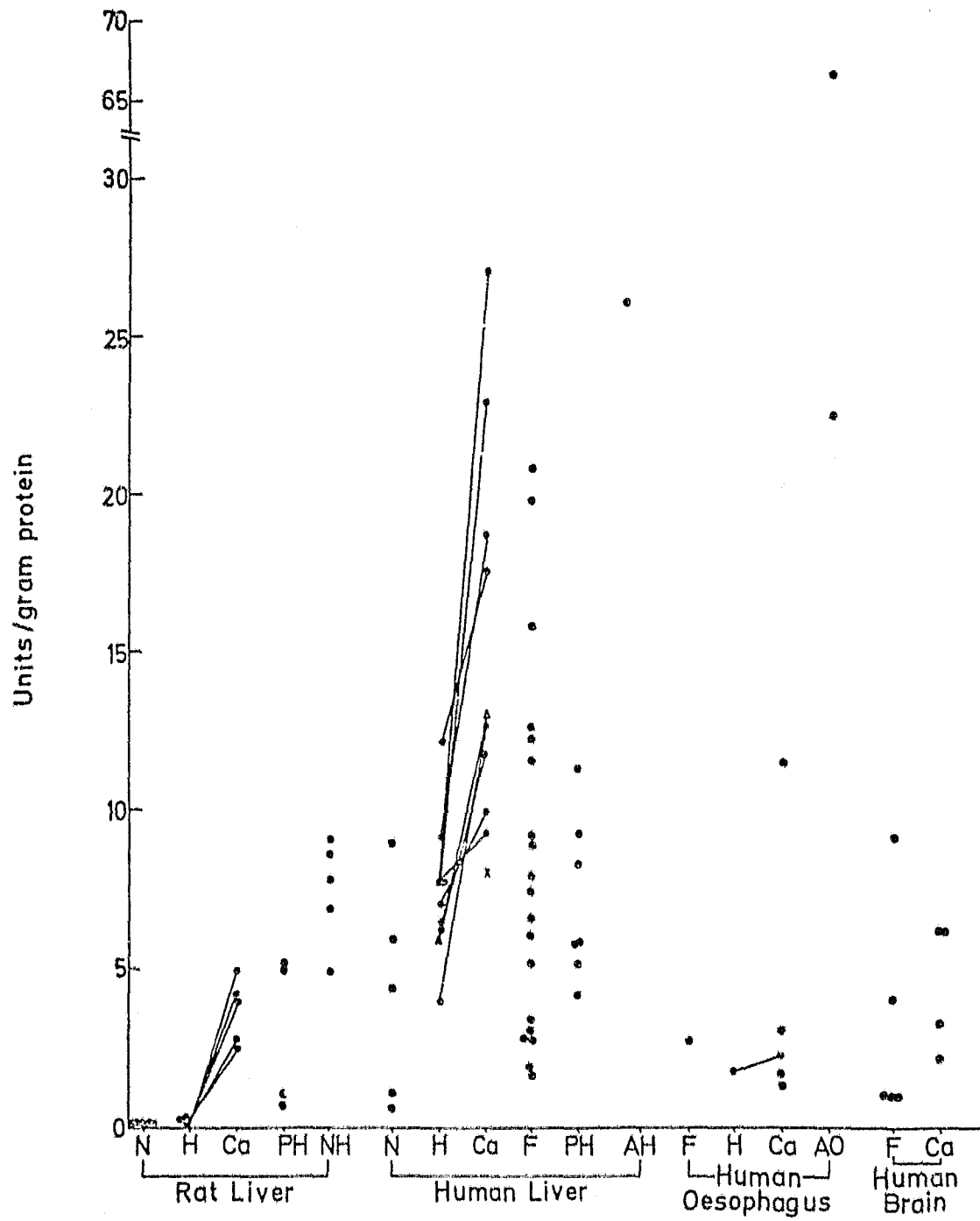


Key: See Figure 3.

FIGURE 9.

87.

DEOXYCYTIDYLATE DEAMINASE ACTIVITY IN RAT AND HUMAN
NORMAL AD. AT, HOST, CANCEROUS AND FOETAL TISSUES, AND
CELL-LINES



Key: See Figure 3.

those in host rat liver were undetectable. Levels of activity of this enzyme were significantly increased in rat transplantable hepatomas.

dTMP synthetase activities in normal and host rat liver tissues were low and did not differ significantly. Levels of this enzyme in the rat transplantable hepatomas were fifty-fold greater than those in host rat liver.

Levels of dCMP deaminase were about two orders of magnitude higher than the other enzymes assayed. It was again found that activities in the rat hepatomas were significantly greater than those in normal or host rat liver tissues.

It is thus clear that the activities of the enzymes of DNA synthesis assayed were significantly increased in transplantable rat hepatomas, when compared with rat normal or host liver. With the exception of dTMP kinase, the levels of the enzymes assayed appeared to be slightly elevated in rat host livers in comparison to those in rat normal liver, although the small number of samples assayed renders these differences statistically insignificant.

The activities of DNA polymerase, Tdr kinase, dTMP kinase, dTMP synthetase, and dCMP deaminase in the NRH and PRH hepatoma cell-lines are summarised in Table 6 and Figures 3 to 9.

It can be seen that the levels of DNA polymerase were higher in the NRH cells than in the PRH cells. Both cell-lines showed a preference for denatured DNA template, although this was much more marked in the case of the NRH cells than in the PRH cells.

Levels of Tdr kinase were very low in the PRH cells, being some 24-fold lower than those in the NRH cell-line.

The mean activities for dTMP kinase in the two cell-lines were

TABLE 6

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA SYNTHESIS
IN THE NRH AND PRH RAT HEPATOMA CELL-LINES

Enzyme	Activity (mUnits/gram protein)*	
	NRH cells	PRH cells
<u>DNA Polymerase</u>		
with native DNA template	63.2 ± 14.8 (5)	30.1 ± 2.6 (4)
with denatured DNA template	95.9 ± 10.6 (5)	35.1 ± 10.9 (4)
Ratio: Activities $\frac{\text{denatured DNA}}{\text{native DNA}}$	1.71 ± 0.22 (5)	1.12 ± 0.29 (4)
<u>Thymidine Kinase</u>	241 ± 55 (5)	10.0 ± 8.9 (4)
<u>Thymidylate Kinase</u>	289 ± 55 (5)	274 ± 163 (4)
<u>Thymidylate Synthetase</u>	34.9 ± 10.6 (5)	8.08 ± 2.47 (4)
<u>Deoxycytidylate Deaminase</u>	7 470 ± 750 (5)	2 890 ± 1 220 (4)

* Results are expressed as a mean ± the standard error of the mean, with the number of samples assayed shown in parentheses.

comparable, but levels in both cell-lines showed considerable variability at different cell harvestings.

The levels of dTMP synthetase and dCMP deaminase were higher in the NRH cell-line.

The levels of activity of the enzymes assayed in the NRH rat hepatoma cell-lines were consistently higher than those in the hepatoma tissue from which they were derived. These findings are in keeping with the very rapid growth rate of this cell-line. On the other hand the fact that the PRH rat hepatoma cell-line was grown under conditions which were known to be sub-optimal for the growth of these cells can be correlated with the lower activities of the enzymes of DNA synthesis in these cells.

Human Adult Normal and Foetal Livers, Hepatomas, and Cell-lines

Derived from Human Hepatomas

Stability of the Enzymes Studied

Stability studies using human foetal liver and hepatoma tissues showed that Tdr kinase and dCMP deaminase were stable to storage at -70°C for periods of up to five months. DNA polymerase, dTMP kinase and thymidylate synthetase were unstable to any form of storage and had to be assayed immediately.

Enzyme Levels in Liver Tissues

The results obtained for levels of these enzymes in human normal adult liver, host liver, hepatoma and foetal liver are shown in Table 7 and Figures 3 to 9. A line is drawn to join the activity in the cancerous tissue with that of the corresponding host tissue. This permits comparison of levels in an individual, thus reducing to a minimum differences due to biological variation, dietary states, effects of drugs, and enzyme changes occurring in the dying patient.

TABLE 7

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA SYNTHESIS IN HUMAN ADULT
NORMAL AND HOST LIVER, HEPATOMA, AND FETAL LIVER

Enzyme	Activity (units/g wet protein) *			Statistical significance of differences in levels **					
	Adult Normal Liver	Host Liver	Hepatoma	Fetal Liver	Normal Liver vs Host Liver	Normal Liver vs Hepatoma	Host Liver vs Hepatoma	Fetal Liver vs Normal Liver	Fetal Liver vs Host Liver
<u>DNA Polymerase</u>									
With native DNA template	0.33±0.15 (5)	1.82±0.4 (11)	4.00±0.7 (12)	7.00±0.91 (20)	P<0.0025	P<0.0005	P<0.01	P<0.0005	P<0.01
With denatured DNA template	0.18±0.11 (5)	1.18±0.19 (11)	5.17±0.95 (12)	17.2±2.6 (20)	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005
Autotransferase activities									
measured with native DNA	0.60±0.35 (4)	0.78±0.12 (11)	1.43±0.27 (12)	2.30±0.20 (20)	N/S	P<0.05	P<0.025	P<0.0005	P<0.01
<u>Thymidine Kinase</u>	11.1±1.6 (5)	11.9±1.5 (11)	12.7±2.2 (12)	137±13.6 (20)	N/S	N/S	N/S	P<0.0005	P<0.0005
<u>Adenylate Kinase</u>	6.00±3.85 (4)	19.4±5.2 (9)	30.5±13.1 (10)	124±27 (20)	P<0.05	P<0.0025	P<0.005	P<0.0005	P<0.005
<u>Adenylate Synthetase</u>	0.07±0.06 (5)	N/D	2.42±0.8 (10)	29.4±6.6 (12)	-	P<0.0005	P<0.0005	P<0.0005	P<0.0005
<u>Deoxythymidylate Synthase</u>	150±20 (10/5)	7420±770 (9)	100±2000 (10/8)	100±130 (10)	P<0.10	P<0.0025	P<0.0025	P<0.05	P<0.005

* Results are expressed as a mean ± the standard error of the mean, with the number of samples assayed shown in parentheses.

** A value of P<0.01 is regarded as representing a significant difference.

N/D = no detectable activity

N/S = not significantly different.

From the Table it can be seen that levels for the DNA polymerase forms using native or denatured template DNA preferentially, increased in the order normal liver < host liver < hepatoma < foetal liver. Whereas DNA polymerases from human normal and host livers showed a preference for native template DNA, the forms predominant in hepatoma and foetal liver showed greater activities with denatured template DNA. Figures 3 and 4 show that, although there was considerable overlap of values for hepatomas and host livers, when individuals are considered there was an increased level in the hepatomas in nearly every case.

Levels of Tdr kinase in normal, host and cancerous human liver were comparable, whereas activity was about ten-fold greater in human foetal liver.

dTMP kinase showed a pattern similar to the DNA polymerases with the lowest mean levels in adult normal liver and the highest in foetal liver. Figure 7 shows that, as for DNA polymerase, despite overlap of values for host livers and hepatomas, the level in the hepatoma from a particular patient was higher than that in the corresponding host liver in all cases but one.

Activities of dTMP synthetase in normal liver and host liver were undetectable or very low. Levels in hepatomas were higher, but still considerably lower than the activities found in foetal liver preparations.

The levels of dCMP deaminase in the human liver tissues were found to be two-to-three orders of magnitude greater than the other enzymes assayed. Similar observations (page 88) were made in the case of rat dCMP deaminase. This is the only enzyme assayed for which levels were higher in human hepatoma tissues than in foetal liver.

With the exception of Tdr kinase, the levels of all of the enzymes assayed were significantly elevated in human hepatoma tissue when com-

pared with those in normal and host liver. It is of interest that the levels of DNA polymerase, dTMP kinase and dCMP deaminase were higher in host liver than in normal liver, although, possibly due to the small numbers of normal livers assayed, only DNA polymerase was statistically significantly higher.

Levels of Enzymes in Foetal Livers at Different Gestational Ages

The levels of human foetal liver Tdr kinase, dTMP kinase, dTMP synthetase, dCMP deaminase, and the forms of DNA polymerase preferring native or denatured DNA templates are plotted in Figure 10 as a function of gestational age. It can be seen that the levels of these enzymes are not constant but increase in activity in three regions: at twelve, eighteen to twenty, and twenty-five weeks gestation. Although all of the enzymes assayed showed this periodicity, they did not all show the same percentage increase in activity.

Enzyme Levels in Human Hepatoma Cell-lines

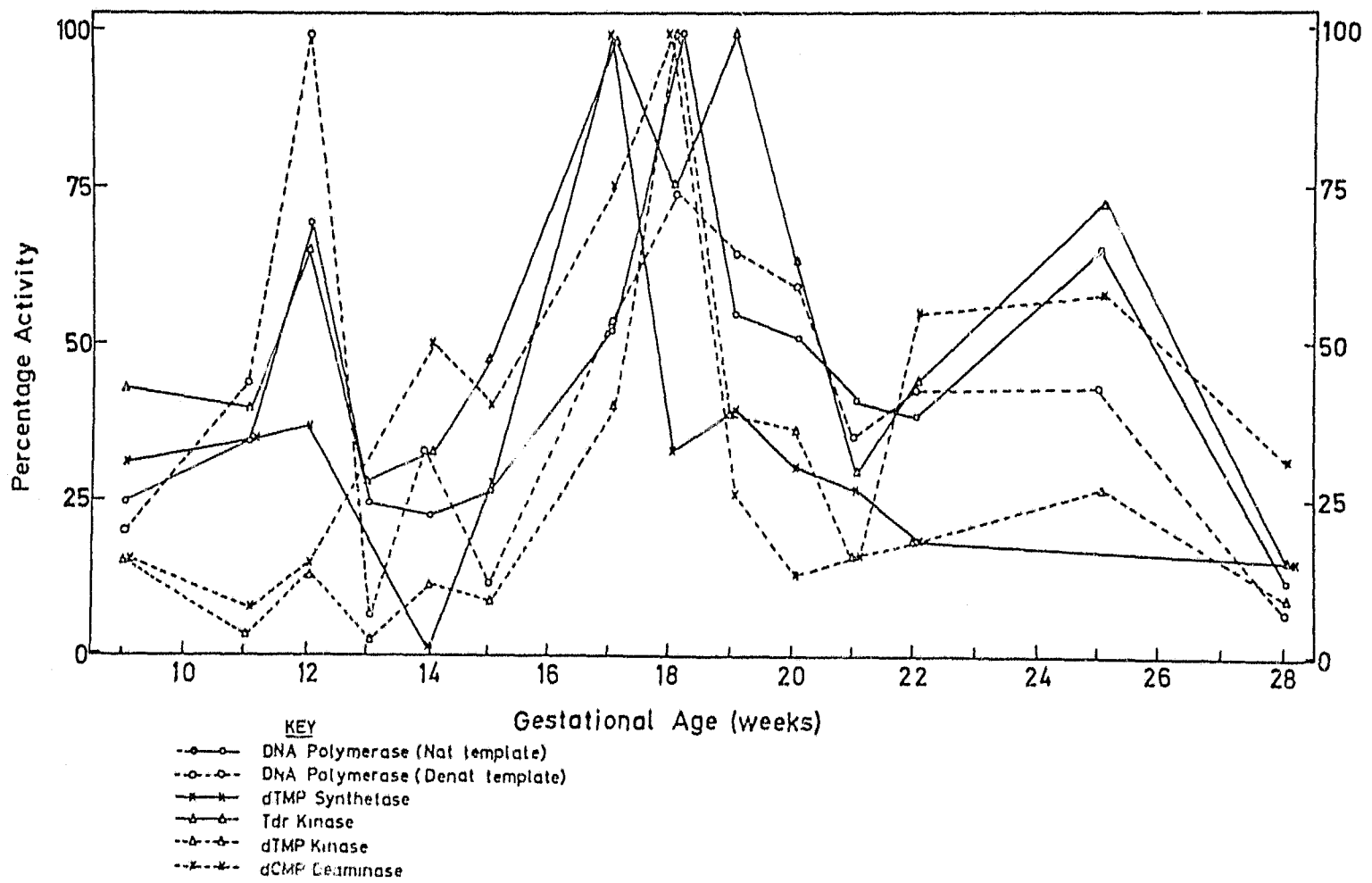
Table 8 compares the levels of some key enzymes of DNA synthesis in the AHH and PHH cell-lines.

It can be seen that DNA polymerase levels with both native and denatured template DNA's were two-fold higher in the PHH cell-line than the AHH cell-line, and that in both cases the predominant form of the enzyme was that which preferred denatured template DNA.

Tdr kinase, dTMP kinase and dTMP synthetase activities were greater in the PHH cell-line than in the AHH cell-line. On the other hand, dCMP deaminase levels were four-fold higher in the AHH cells than in the PHH cells.

The levels of the enzymes in the slow-growing AHH cell-line were, on the whole, comparable with the levels measured in human hepatoma tissue, whereas those in the PHH cell-line correlated well with the known rapid growth rate of this cell-line.

FIGURE 10



ACTIVITIES OF KEY ENZYMES OF DNA SYNTHESIS IN THE LIVERS
OF HUMAN FOETUSES OF DIFFERENT GESTATIONAL AGES.

TABLE 8

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA
SYNTHESIS IN AHH AND PHH HUMAN HEPATOMA CELL-LINES

Enzyme	Activity (mUnits/gram protein)*			
	AHH cells		PHH cells	
<u>DNA Polymerase</u>				
with native DNA template	9.00	(1)	17.0 ± 1.5	(7)
with denatured DNA template	16.6	(1)	30.6 ± 3.0	(7)
Ratio: Activities $\frac{\text{denatured DNA}}{\text{native DNA}}$	1.84	(1)	1.81 ± 0.12	(7)
<u>Thymidine Kinase</u>	10.7	(1)	317 ± 47	(7)
<u>Thymidylate Kinase</u>	149	(1)	462 ± 67	(7)
<u>Thymidylate Synthetase</u>	0.76	(1)	35.2 ± 11.9	(5)
<u>Deoxycytidylate Deaminase</u>	26 100	(1)	7 100 ± 980	(7)

* Results are expressed as a mean ± the standard error of the mean, with the number of samples shown in parentheses.

Human Foetal, Host and Cancerous Oesophageal Tissue, and an
Oesophageal Cancer Cell-line

Table 9 shows a comparison of the levels of DNA polymerase, Tdr kinase, dTMP kinase, dTMP synthetase and dCMP deaminase in human foetal, host and cancerous oesophageal tissue, and in a human oesophageal cancer cell-line.

Levels of the DNA polymerase forms preferring both native and denatured template DNA were comparable in foetal and host oesophageal tissue and were only 2-to 3-fold higher in cancerous oesophageal tissue. In the AHO cell-line levels were about 10-to 20-fold higher than those in cancerous tissue. In all cases the form of the enzyme showing a preference for denatured template DNA predominated.

The levels of Tdr kinase increased in the order foetal < host < oesophageal cancer < AHO cell-line. Activity in the AHO cells was seven-fold greater than in oesophageal cancer tissue.

In the case of dTMP kinase, levels in foetal and cancerous oesophageal tissue were lower than in the single specimen of host tissue assayed. High levels of the enzyme were found in the AHO cell-line.

Levels of dTMP synthetase were undetectable in host oesophageal tissue. Relatively low levels were measured in foetal and tumorous oesophageal tissue and the activity in the AHO cells was some two-fold greater than in the tumour tissue.

Levels of dCMP deaminase increased in the order host < foetal < Ca oesophagus <<< AHO cells. The levels of this enzyme in the AHO cells were amongst the highest measured in the present study. As was the case for human liver tissues, it was found that levels of dCMP deaminase were generally two- to three-orders of magnitude higher than those of the other enzymes assayed.

TABLE 9

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA SYNTHESIS IN HUMAN FOETAL, HOST
AND CANCEROUS OESOPHAGEAL TISSUE, AND IN A HUMAN OESOPHAGEAL CANCER CELL-LINE (AHO)

Enzyme	Activity (mUnits/gram protein)*			
	Foetal Oesophagus	Host Oesophagus	Ca Oesophagus	AHO cells
<u>DNA Polymerase</u> with native DNA template with denatured DNA template Ratio: Activities $\frac{\text{denatured DNA}}{\text{native DNA}}$	1.17 (1)	1.06 (1)	3.07 $\pm$ 0.92 (5)	29.6 $\pm$ 2.25 (2)
	2.00 (1)	2.17 (1)	3.85 $\pm$ 1.37 (5)	69.6 $\pm$ 6.15 (2)
	1.71 (1)	2.05 (1)	1.24 $\pm$ 0.36 (5)	2.38 $\pm$ 0.39 (2)
<u>Thymidine Kinase</u>	5.67 (1)	13.2 (1)	34.4 $\pm$ 7.7 (5)	230 $\pm$ 126 (2)
<u>Thymidylate Kinase</u>	8.00 (1)	83.4 (1)	45.3 $\pm$ 16.1 (5)	285 $\pm$ 16 (2)
<u>Thymidylate Synthetase</u>	1.33 (1)	N/D (1)	1.94 $\pm$ 1.02 (5)	5.39 $\pm$ 0.39 (2)
<u>Deoxycytidylate Deaminase</u>	2 730 (1)	1 740 (1)	3 940 $\pm$ 1 910 (5)	45 000 $\pm$ 23 000 (2)

* Results are expressed as a mean $\pm$ the standard error of the mean, with the number of samples assayed shown in parentheses.

N/D = no detectable activity.

The pattern of increased activity of DNA polymerase, dTMP kinase, dTMP synthetase and dCMP deaminase in human normal and cancerous liver tissues was generally not well demonstrated by the mean levels measured in normal and cancerous oesophageal tissues. However in the single matched oesophageal host-tumour pair (Figures 3 to 9) it can be seen that, with the exception of dTMP kinase, levels of the enzymes assayed were elevated in the cancerous tissue.

Thymidine kinase levels in cancerous oesophageal tissues were increased in contrast to the finding of normal levels for this enzyme in human hepatoma tissue.

Human Meningioma and Human Foetal Brain

Due to the availability of human meningioma tissue it was thought of interest to assay the levels of the 5 enzymes of DNA synthesis in this tissue. Normal meninges were not available. In addition the enzymes were assayed in human foetal brain cortex, although it was realised that this was not analogous to the parent tissue from which the meningioma was derived.

Table 10 shows the levels of DNA polymerase, Tdr kinase, dTMP kinase, dTMP synthetase and dCMP deaminase in human meningioma and foetal brain.

DNA polymerase activities with native and denatured DNA templates were lower in meningiomas than in the foetal brains. In both tissues the form of the enzyme showing a preference for denatured DNA template predominated.

Levels of Tdr kinase and dTMP kinase were respectively two- and ten-fold higher in foetal brain than in meningioma tissue.

No dTMP synthetase activity was detectable in meningioma tissue.

TABLE 10

A COMPARISON OF THE LEVELS OF KEY ENZYMES OF DNA SYNTHESIS
IN HUMAN FETAL BRAIN AND MENINGIOMA TISSUE

Enzyme	Activity (mUnits/gram protein)*	
	Human Fetal brain	Human Meningioma
<u>DNA Polymerase</u>		
with native DNA template	15.8 ± 1.96 (5)	3.21 ± 0.70 (4)
with denatured DNA template	22.5 ± 6.01 (5)	4.92 ± 1.06 (4)
Ratio: Activities $\frac{\text{denatured DNA}}{\text{native DNA}}$	1.52 ± 0.50 (5)	1.55 ± 0.12 (4)
<u>Thymidine Kinase</u>	22.5 ± 6.8 (5)	10.3 ± 3.4 (4)
<u>Thymidylate Kinase</u>	201 ± 40 (5)	23.6 ± 3.1 (4)
<u>Thymidylate Synthetase</u>	5.76 ± 3.53 (5)	N/D (4)
<u>Deoxycytidylate Deaminase</u>	3 250 ± 1 590 (5)	4 480 ± 1 030 (4)

* Results are expressed as a mean ± the standard error of the mean,
with the number of samples assayed shown in parentheses.

N/D = no detectable activity.

Levels of dCMP deaminase in foetal brain and meningioma were comparable.

The levels of DNA polymerases and Tdr kinase in human meningiomas were similar to the levels of the corresponding enzymes in human hepatomas. Levels of dTMP kinase and dCMP deaminase were respectively two- to three-fold, and three- to four-fold higher in human hepatomas than in human meningiomas. dTMP synthetase activity which was present at low levels in hepatomas was not detectable in the meningiomas.

DISCUSSION

It is desirable to undertake studies on human tissue in order to check the validity of the extrapolation of results using other animals (eg. the rat) to the human situation. For example, drug regimens are frequently based on the blocking of enzymes which catalyse key metabolic steps in DNA synthesis in animal tissues or tumours. There are, however, difficulties and disadvantages associated with the use of human tissues. These include limited availability, uncontrolled variability due to age, sex, diet and pathology, and delays in obtaining tissues after death. By comparing apparently uninvolved parent tissue from the same patient from whom the tumour is obtained, at least some of these variables can be reduced.

Although a number of the hepatoma patients had received chemotherapy aimed at blocking DNA synthesis, levels of the enzymes assayed in hepatomas of these patients were found to be in a range similar to those in patients who had received no treatment. Death had generally intervened in the initial stages of chemotherapy. It was hoped that levels of the enzymes of DNA synthesis in biopsied samples of tissue might be investigated before and during treatment. However the risks to the patients of such investigations could not be justified.

ASSAY METHODS

Assay methods for DNA polymerase, Tdr kinase, dTMP kinase, dTMP synthetase and dCMP deaminase were modified so that assay conditions were optimal for human foetal and hepatoma tissues. Saturating levels of substrates were used and reaction rates were linear during the assay incubation periods. The use of isotopically-labelled substrates allowed sufficient sensitivity for the measurement of the very low levels of activity of some of these enzymes (eg. dTMP synthetase) in human adult normal liver.

In the case of DNA polymerase, Ove *et al.* (1969a) found that the enzyme forms characteristic of non-dividing adult liver tissue and of rapidly-dividing foetal, regenerating or neoplastic liver could be distinguished by their preferences for, respectively, native and denatured template DNA. In the present study DNA polymerase levels of each tissue preparation were measured using both types of template DNA, and the major form of the enzyme activity was determined from the ratio of the enzyme activities using the two types of DNA template.

The isotopic and spectrophotometric methods for the measurement of dCMP deaminase activity gave comparable results when used to measure the levels of activity in extracts from rapidly dividing human or rat liver tissues.

STABILITY OF THE ENZYMES ASSAYED FROM HUMAN TISSUES

DNA polymerases from human foetal, normal and cancerous liver tissues were unstable to storage at both -20°C and -70°C , confirming the finding of instability of this enzyme from rat regenerating liver by Mantsavinov (1964). Similarly dTMP synthetase was unstable to storage at these temperatures, a finding which is in agreement with that for the enzyme from Ehrlich ascites cells (Hartmann and Heidelberger, 1961). The activity of human liver dTMP kinase was also found to decrease on storage at these temperatures, which is in agreement with the instability on storage reported for the enzyme from human leukaemic blast cells (Lee and Cheng, 1977). On the other hand, the enzyme from rat liver is stable to storage (Bukovsky and Roth, 1965). Tdr kinase did not lose activity on storage of human liver tissues at -20°C , a finding which differs from that for the rat liver enzyme (Bresnick and Burleson, 1970; Adelstein *et al.*, 1971). dCMP deaminase activity in the human liver tissues was stable to storage at -20°C for several months. However it was necessary

to extract the enzyme activity using homogenising medium containing 0.5mM dCTP. The latter finding is in agreement with that of Sneider and Potter (1968) for the enzyme from rat hepatomas.

STUDIES OF LEVELS OF SOME ENZYMES OF DNA SYNTHESIS IN HUMAN
AND RAT TISSUES AND CELL-LINES

The activity of ribonucleotide reductase has been shown to correlate well with growth-rate in rat transplantable hepatomas (Elford *et al.*, 1970). It was originally intended that measurement of the levels of activity of this enzyme would be included in the present study. However it was found that ribonucleotide reductase activity was very unstable and that the assay procedure (as described by Reichard, 1972) was too time-consuming to allow the assay of this enzyme in parallel with other enzymes of DNA synthesis. Assays for this enzyme were therefore not included in the present study.

Rat Normal and Host Livers, Hepatomas, and Hepatoma Cell-lines

The levels of the key enzymes of DNA synthesis were found to be low in rat normal liver, as found by others (Ferdinandus *et al.*, 1971; Sneider *et al.*, 1969; Weber, 1974).

DNA polymerase activity showed a preference for native DNA as a template, confirming the results for rat liver DNA polymerase of Iwamura *et al.* (1968), Ove *et al.* (1969a & b) and Baril and Laszlo (1970).

Levels of dCMP deaminase were at least an order of magnitude greater than the levels of the other enzymes assayed in the present study.

The activities of the enzymes assayed in the livers of rats bearing transplantable hepatomas (host livers) were also found to be low. However, with the exception of dTMP kinase activity, which could not be detected, the ranges of activity for each of the enzymes appeared to be slightly elevated in comparison with the levels of the corresponding

enzymes in rat normal liver (Table 5). This suggests that there may be some tumour-induced, possibly serum-mediated, effect on the enzymes of DNA synthesis in the host livers of hepatoma-bearing rats, as has also been proposed by Shirasaka and Fujii (1975). The absence of dTMP kinase activity in rat host livers differs from the findings of these authors, however, since they observed that levels of this enzyme were increased in the host livers of rats bearing Yoshida sarcoma tumours. Failure to measure dTMP kinase activity in rat host liver in the present study was not due to faulty reagents or assay conditions since high levels of this enzyme were found in the corresponding hepatoma tissues which were assayed simultaneously.

The levels of the enzymes assayed in rat transplantable hepatoma tissue showed statistically significant increases over the levels of these enzymes in rat normal liver.

Levels of the form of DNA polymerase which prefers denatured template DNA increased to a greater extent than those of the form using native template DNA, so that the former became the predominant form in the rat hepatomas. This finding is in agreement with those of Iwamura *et al.* (1968), Ove *et al.* (1969a) and Baril and Laszlo (1970) for neoplastic rat liver tissues.

It can be seen from Table 11 that the activities of these enzymes of DNA synthesis in the 3' Me-DAB-induced rat hepatoma corresponded with those reported by Weber (1974) for a rapidly-growing Morris 3683F rat hepatoma. The rapid growth rate of the hepatoma used in the present study is attested to by the observation that death of the host rats bearing the 3' Me-DAB hepatomas occurred within three to four weeks after implantation of the tumour. The elevated levels of these enzymes of DNA synthesis in the rat hepatoma tissues suggest that both the *de novo* and salvage path-

TABLE 11
RATIOS OF ACTIVITIES OF DNA SYNTHESIS IN
RAT HEPATOMAS TO THOSE IN RAT NORMAL LIVER

Enzyme	3' Me-DAB Hepatoma	3683F Hepatoma (from Weber, 1974)
DNA polymerase (denatured DNA template)	25	58
Tdr kinase	50	100
dTMP kinase	105	(approx 71*)
dTMP synthetase	70	29
dCMP deaminase	70	8

* No value is given for dTMP kinase in the 3683F hepatoma.

However the activity appears to be similar to that listed in the same reference for the rat Novikoff hepatoma. Results for this hepatoma are therefore listed here in place of the 3683F hepatoma.

ways leading to dTTP and DNA synthesis are markedly elevated in this rapidly-growing tissue.

The activities obtained for levels of the enzymes of DNA synthesis in the rat hepatoma cell-lines showed scatter over a wide range. It can however be seen that, with the exception of dTMP synthetase, levels of these enzymes in the NRH cell-line were higher than in the tumour tissue from which the cell-line was derived. These cells had a doubling time of 22 hours and were dedifferentiated (Albrecht *et al.*, 1972); the high levels of the enzymes of DNA synthesis thus correlate well with the rapid-growth rate of these cells. The very high levels of dTMP kinase measured in the NRH cells are in agreement with the correlation between growth rate and dTMP kinase activity in Chang liver cells (Eker, 1968).

The PRH cell-line was cultured under conditions which were optimal for the growth of the PHH human hepatoma cell-line. It was found that these conditions were not optimal for the growth of the PRH cell-line and that the growth rate of these cells was slower than that of the NRH cell-line. Cells were harvested at approximately the time of confluency, and it was found that the levels of the enzymes assayed showed a wide variation at different cell-harvestings. Generally the range of levels of the enzymes assayed was similar to those found for rat hepatoma tissue, with the notable exception of Tdr kinase. The very low levels measured for this enzyme suggest that the salvage of thymidine plays a relatively insignificant role in DNA metabolism in the PRH cells.

A possible explanation for the wide ranges of enzyme levels obtained for both the NRH and PRH cell-lines is that, despite attempts to harvest cells just before the time of confluency, the actual growth rates of the cells at the time of harvesting may have varied. In this respect

it has been shown that cellular DNA synthesis and the levels of key enzymes involved in the production of DNA in human HeLa and Chang cell-lines vary according to the growth stage of the cells (Brent *et al.*, 1965 and Eker, 1968).

Human Normal and Host Livers, and Hepatomas

The levels of the enzymes DNA polymerase and dTMP synthetase in human normal liver were low or, at times, undetectable, suggesting that, as in rat liver, there is little or no DNA synthesis in differentiated human normal liver. DNA polymerase activity was greatest when native template DNA was used. It is of interest that the levels of Tdr kinase and dCMP deaminase were notably higher in adult human liver tissue than in the corresponding rat tissue. High levels of dCMP deaminase in human adult normal liver have been described by Giusti *et al.* (1970). These workers reported levels which were four-fold greater than those measured in the present study, but it should be noted that they used an assay based on the measurement of ammonia released, which would be less specific than the isotopic assay used in the present study. The metabolic significance of the elevated Tdr kinase and dCMP deaminase activities in human normal liver is at present not clear.

In human host liver DNA polymerases, using either native or denatured template DNA's, showed statistically significant increases in activity. The ratios of the enzyme activities using the two types of template DNA suggest that the DNA polymerase using native template DNA preferentially is still the dominant form of the enzyme in this tissue.

Levels of Tdr kinase activity in human host livers were in the same range as those for the enzyme from normal livers. The range of values for Tdr kinase activity in human host liver was comparable with that

measured in human kidney tissue adjacent to hypernephromas by Gordon *et al.* (1968).

The activities of dTMP kinase in human host livers were about three-fold higher than the levels of this enzyme in human normal adult liver. The levels of dTMP kinase were again comparable with those measured in kidney tissue adjacent to hypernephromas by Gordon *et al.* (1968).

The levels of dCMP deaminase in human host livers appeared to be elevated in comparison with human normal livers, although the differences were not statistically significant.

The finding of increased DNA polymerase and dTMP kinase activities in human host liver is similar to the increases in these enzymes reported by Shirasaka and Fujii (1975) for livers in rats bearing Yoshida sarcomas. The elevated DNA polymerase, dTMP kinase and dCMP deaminase activities in human host liver may also reflect regeneration or repair of damaged cells in this tissue. In this respect it has been shown that dCMP deaminase is elevated in most acute liver conditions in humans (Miller and Ressler, 1969). If this is the case the absence of dTMP synthetase in human host liver suggests that, during the regeneration or repair processes, dTMP is produced primarily via the Tdr kinase salvage pathway or possibly via a dCMP deaminase-catalysed conversion of 5-methyl-dCMP to dTMP, as is discussed on page 114.

Levels of DNA polymerase in human hepatomas were significantly higher than those in normal or host liver. DNA polymerase activities showed a preference for denatured template DNA, a finding which is in agreement with that for rat transplantable hepatomas in the present study.

Tdr kinase levels showed no significant difference from those in human normal or host liver. These results are in agreement with those of

Gordon *et al.* (1968) for human lung carcinomas, and Laszlo *et al.* (1969) for human colonic cancer. The absence of any increase in Tdr kinase activity in human neoplastic liver is the most striking difference between human hepatoma tissues and the rat transplantable hepatoma. Failure to show increased Tdr kinase activity is not a property of all human cancerous tissues, since the levels of this enzyme have been shown to increase in human carcinomas of the caecum, rectum, tonsil, tongue, larynx, mammary gland, and in bone sarcomas; and to decrease in hypernephromas, relative to the corresponding host tissues (Gordon *et al.*, 1968).

Levels of dTMP kinase were significantly increased in human hepatoma in comparison with normal or host livers. Similar increases in dTMP kinase activity have been reported for human carcinomas of the caecum, rectum, uterus, tonsil, tongue, larynx, mammary gland and lung, and for bone sarcomas and hypernephromas (Gordon *et al.*, 1968).

Measurable dTMP synthetase activity was present in human hepatoma tissue, confirming that the *de novo* pathway of dTMP synthesis is operative in human cancerous liver. Levels of the enzyme were however very low in comparison with those in rat hepatoma.

Significantly higher levels of dCMP deaminase were found in human hepatoma compared with human normal or host livers. This finding is in agreement with those of Giusti *et al.* (1970) who found that dCMP deaminase activity was increased, relative to the corresponding non-malignant tissues, in human epidermoid and basal cell carcinomas of the skin, and in carcinomas of the uterus and ovary. These authors also reported high levels of dCMP deaminase in human malignant melanomas, breast and lung carcinomas, adenocarcinomas of the colon and rectum, and in a malignant neuroma.

As can be seen from Figures 3 to 9 the levels of the enzymes in surgically-removed or biopsied human hepatoma tissues were in the same range as the enzymes assayed in tissues obtained at autopsy. These findings confirm that, although some of the enzymes are unstable to storage (page 102), the rapid removal of the autopsy tissues and rapid preparation of the tissues for assay permitted reliable estimates of enzyme activity in the autopsied liver tissues.

The levels of these key enzymes of DNA synthesis in human hepatomas were, with the exception of Tdr kinase, increased relative to human normal or host liver tissues. On the other hand, the relatively slight increases in the levels of these enzymes in comparison with those in the rat hepatoma suggest that the human hepatomas are characterised by a slower growth rate. This observation is in agreement with the recent findings that the incorporation of Tdr into DNA in human hepatoma tissue was not increased, in comparison with human normal liver, whereas the catabolism of Tdr was slightly decreased (Weber *et al.*, 1976b). These workers concluded that human hepatoma tissues showed a 'low to medium' malignancy and growth rate. The finding by Weber *et al.* (1976b) that the incorporation of Tdr into DNA in human hepatomas is comparable with that in normal liver tissue may be explained by the finding of the present study that Tdr kinase activity is not increased in human cancerous liver.

Human Foetal Livers

Differences in the enzyme levels assayed in human hepatoma tissue suggested that the pattern of DNA metabolism in this tissue was altered in comparison with host or normal liver. Since Potter (1968) has suggested that the metabolic pattern of hepatomas may resemble that of foetal liver cells in a state of blocked maturation, the levels of key enzymes of

DNA synthesis were assayed in a series of human foetal livers. The levels of all of the enzymes, with the exception of dCMP deaminase, were found to be significantly higher than those in human hepatoma tissue. Activities of dCMP deaminase were significantly lower in human foetal liver than in cancerous liver. The finding of very high levels of Tdr kinase in foetal liver suggests that the salvage pathway is active in human foetal liver. The levels of Tdr kinase were much lower in human hepatoma tissue when compared to human foetal liver and this would suggest, according to Potter's theory of oncogeny as blocked ontogeny (Potter, 1965), that the human hepatoma cells more closely resemble differentiated adult liver cells rather than human foetal liver cells. It is however important to note that foetal liver is primarily a haemopoietic organ and that the relative proportions of haemopoietic to parenchymal cells at the different stages of gestation are not known. The present study does not distinguish between the contributions of these two cell types.

The levels of the key enzymes of DNA synthesis were not constant at all stages of foetal development, but appeared to show consistent alterations in activity at different gestational ages (Figure 10). This variation suggests that specific peak periods of DNA synthesis occur in foetal liver during gestation. Periodic DNA synthesis has been reported during the development of rat foetal brain (Winick, 1968; Fish and Winick, 1969) and during the erythropoietin-stimulated transformation of cultured mouse spleen cells (Kodman *et al.*, 1976). Mukherjee and Hastings (1973) have shown that in human foetal liver, during the period of eight to fifteen weeks gestation, there is a decrease in Tdr incorporation into DNA at twelve weeks followed by an increase to the eight-week levels of Tdr incorporation by fifteen weeks gestation. In contrast

to these findings, the observations of the present study suggest that the activities of the key enzymes of DNA synthesis increased at twelve weeks gestation, with decreases at fourteen to fifteen weeks, and subsequent increases between weeks eighteen to twenty. Studies of DNA synthesis in developing human foetal liver would need to be concurrently undertaken with assays for the levels of the key enzymes of DNA synthesis to clarify the relationship between the enzyme levels and synthesis of DNA.

Human Hepatoma Cell-lines

Levels of the enzymes assayed in the AHH cell-line were in the same range as those in human hepatoma tissues. The cell-line is characterised by a very slow growth rate, this in fact being the reason why adequate material for the assay of the enzymes of DNA synthesis could only be obtained on a single occasion. Of particular interest are the findings in the AHH cell-line of low Tdr kinase and high dCMP deaminase activities which are also characteristic of human hepatoma tissues.

In contrast to the slow growth rate of the AHH cell-line, the PHH cells showed a very rapid growth rate. This was also reflected by the levels of the enzymes of DNA synthesis. Although these showed a wide scatter, they were (with the exception of dCMP deaminase) higher than the corresponding ranges of activity in human hepatoma tissues. In general the PHH cell-line appears to represent a selection for a rapidly-growing cell population which is not truly representative, in terms of DNA metabolism, of human cancerous liver tissues.

The results of studies on the levels of these enzymes in human hepatoma cell-lines suggest that care should be taken in interpreting results obtained with these cell-lines, since it appears possible that

rapidly growing cell types which are not representative of the parent tissue may be selected during the establishment of the line.

Human Foetal, Host and Cancerous Oesophageal Tissues, and an Oesophageal Carcinoma Cell-line.

Levels of the enzymes assayed in human foetal oesophageal tissue were relatively low in comparison with those in human foetal liver. With the exception of dTMP kinase, higher levels of activity were found in the cancerous oesophageal tissue than in a segment of apparently normal oesophagus biopsied from a site adjacent to the cancer. Enzyme levels in the tumour tissue were, with the exception of dCMP deaminase, in a similar range to those in human hepatoma tissue, suggesting that tumour specific alterations in DNA synthesis also occur in oesophageal cancer.

DNA polymerase activities assayed in host oesophageal tissue showed a marked preference for denatured template DNA. This may be due to the fact that the basal cells in this tissue are constantly dividing to replace cells lost at the mucosal surface.

The levels of the enzymes assayed in the AHO cell-line were very high, particularly in the case of dCMP deaminase. Enzyme activities in these cells showed little similarity to those in the human cancerous tissue from which the cell-line had been derived. This suggests that selection for a very rapidly growing cell-type has occurred during the development of the AHO cell-line.

Human Foetal Brain and Meningiomas

Relatively high levels of the enzymes of DNA synthesis were measured in human foetal brain (cortex) and meningioma tissues, the only exception being that no dTMP synthetase activity could be detected in the meningioma tissues. It may be argued that, because of the absence of dTMP synthetase, salvage pathways leading to DNA synthesis are of

greater importance in human meningioma tissue than the *de novo* pathway. The results obtained in the present study correlate with a slow tumour growth rate and a low or absent malignancy. This is in agreement with the clinical finding that meningiomas are benign tumours.

The Role of Deoxycytidylate Deaminase in dTMP Synthesis

dCMP deaminase is generally accepted to be responsible for the conversion of deoxycytidylate to deoxyuridylate. However, the obligatory role of dCMP deaminase in dUMP synthesis has been questioned by Sneider and Potter (1969) with the suggestion that the enzyme might provide a direct route to dTMP via the deamination of 5-methyl-dCMP. The work of other groups (eg. Tosi et al., 1972) suggests that a small percentage of the thymine residues in the DNA of sea urchin embryos is derived from the deamination of 5-methylcytosine residues in DNA. In the present study (Table 4) a rat hepatoma preparation was found to deaminate 5-methyl-dCMP three times faster than dCMP. In human liver and hepatoma preparations comparable rates of deamination were measured using either substrate. It would be of interest to study the role of dCMP deaminase in DNA metabolism in human liver tissue, in order to ascertain the significance of the very high levels of the enzyme in this tissue. The possibility of an alternative pathway of dTMP synthesis via this enzyme remains open, but levels of 5-methylcytidine in human cells, or the presence of enzymes capable of producing this substrate, remain to be investigated.

CONCLUSIONS

Of the five enzymes of DNA synthesis studied, the activities of four were found to be elevated both in human and rat hepatomas, relative to the corresponding host livers. The increased activities in the rapidly growing, poorly-differentiated rat hepatomas were considerably

greater than in the human hepatomas, suggesting that the latter tumours have a slower growth-rate, at least at the stage at which material used in the present study was obtained.

The finding that levels of Tdr kinase in human normal and cancerous liver are comparable, suggests that DNA synthesis in the hepatoma cells may not be selectively blocked with the antimetabolite drug 5-fluoro-uracil.

The very high levels of dCMP deaminase in human liver are of interest, especially in the light of the fact that 5-methyl-dCMP is a good substrate for the enzyme. If sufficient amounts of this substrate were available this enzyme could represent a by-pass of dTMP synthesis via dTMP synthetase, one of the pathways of DNA synthesis at which chemotherapy is most frequently directed. Further studies of dCMP deaminase aimed at identifying drugs capable of blocking this enzyme should be considered in the future.

Further studies on Tdr kinases from human liver and hepatoma tissues are described in Chapter 3.

SUMMARY

1. The levels of the enzymes DNA polymerase, thymidine kinase, thymidylate kinase, thymidylate synthetase, and deoxycytidylate deaminase have been measured in rat and human normal and neoplastic liver, in human foetal liver, in cell-lines derived from human hepatomas, and in a rat transplantable hepatoma.
2. The levels of all five enzymes were increased in the rat transplantable hepatoma relative to rat normal or host liver, to a degree which corresponded to the rapid growth-rate of this tumour.
3. With the exception of thymidine kinase, which did not change, the levels of these enzymes were increased in human hepatoma relative to human normal or host liver. The increases were small in comparison with those found in the rapidly-growing rat transplantable hepatoma, suggesting that the human liver tumours have a relatively slow growth-rate.
4. Levels of deoxycytidylate deaminase in both human and rat liver tissues were two to three orders of magnitude higher than the other enzymes assayed.
5. Deoxycytidylate deaminase from human and rat livers and hepatomas could use 5-methyl-deoxycytidylate as a substrate to a similar or greater degree than the natural substrate deoxycytidylate, suggesting a possible alternative pathway of deoxythymidylate production.
6. The activities of these key enzymes of DNA synthesis appeared to vary consistently as a function of gestational age in human foetal liver, suggesting that peaks of DNA synthesis occur at certain fixed periods.

7. The levels of these enzymes in a slow-growing cell-line derived from a human hepatoma were similar to those in human hepatoma tissue. In the case of rapidly growing cell-lines derived from rat and human hepatomas, enzyme levels were generally considerably higher than those in the tissues from which the cell-lines were derived.

CHAPTER 3

PURIFICATION AND CHARACTERISATION OF THYMIDINE
KINASES FROM HUMAN NORMAL LIVER AND HEPATOMA

	<u>Page</u>
<u>MATERIALS AND METHODS</u> -----	121
<u>MATERIALS</u> -----	121
Chemical Reagents and Chromatographic Materials -----	121
Chemicals -----	121
Radiochemicals -----	121
Other Chemicals and Reagents -----	121
Chromatographic Materials -----	121
Human Tissues -----	123
Human Adult Liver -----	123
Human Hepatomas -----	123
<u>METHODS</u> -----	123
Initial Assay Procedures -----	123
Thymidine Kinase -----	123
Thymidylate Kinase -----	123
Thymidylate Phosphatase -----	124
Adenosine Triphosphatase -----	124
Thymidine Analogue Studies -----	125
Protein Determination -----	126
Units of Enzyme Activity -----	126
Methods Adapted to Reduce ATPase Activity in Thymidine Kinase Assays -----	126
Partial Purification of Thymidine Kinases from Human Adult Liver and Hepatoma - Preliminary Studies -----	129
Ammonium Sulphate Fractionation -----	129
Preparation of a High-speed Supernatant Fraction -----	129
Stabilisation of Thymidine Kinase Activity -----	130
Final Purification Procedure for Purification of Thymidine Kinases from Human Adult Liver and Hepatoma -----	130
Extraction of Thymidine Kinase Activity -----	130
DEAE-cellulose Chromatography -----	130
Phosphocellulose p11 Chromatography -----	132

	<u>Page</u>
Further Fractionation of Tdr K II using Gel Filtration on Biogel A1.5 -----	136
Further Purification of Tdr K I using CM-Sephadex C50 Chromatography -----	136
Further Purification of Tdr K IA using Gel-filtration on Biogel A1.5 -----	140
Estimation of the Molecular Weight of Thymidine Kinases Using a Calibrated Biogel A1.5 Column -----	140
Determination of Kinetic Parameters -----	142
<u>RESULTS</u> -----	143
Purification and Characterisation of Thymidine Kinases From Human Normal Liver and Hepatoma -----	143
Purification of Human Liver and Hepatoma Thymidine Kinases -----	143
Characterisation of Human Liver and Hepatoma Thymidine Kinases -----	147
Molecular Weights -----	147
Magnesium Requirements -----	151
pH Optimum Studies -----	151
Thermostability Studies -----	151
Ammonium Sulphate Inhibition -----	154
Michaelis Constants for Thymidine -----	154
Michaelis Constants for Mg-ATP -----	163
Nucleoside and Deoxynucleoside Triphosphates as Phosphate Donors -----	163
Nucleotide and Deoxynucleotide Inhibitors -----	163
Thymidine Analogue Studies -----	166
<u>DISCUSSION</u> -----	170
Purification and Characterisation of Thymidine Kinase Forms from Human Liver and Hepatoma Tissues -----	170
Pilot Purification Studies -----	170
Attempts to Reduce ATPase Contamination -----	172
Purification of Tdr Kinases from Human Normal Liver and Hepatoma -----	173
Characterisation of the Tdr Kinase Forms from Human Normal Liver and Hepatoma -----	174
Molecular Weights -----	174
Magnesium Requirements -----	175

	<u>Page</u>
pH Optimum Studies -----	176
Thermostability Studies -----	177
Ammonium Sulphate Inhibition -----	177
Kinetic Constants for Thymidine -----	178
Phosphate Donor Studies -----	179
Nucleotide Inhibitor Studies -----	181
Thymidine Analogue Studies -----	183
CONCLUSIONS -----	186
SUMMARY -----	188

CHAPTER 3

PURIFICATION AND CHARACTERISATION OF THYMIDINE KINASES FROM HUMAN NORMAL LIVER AND HEPATOMA

MATERIALS AND METHODS

MATERIALS

Chemical Reagents and Chromatographic Materials

Chemicals

In addition to chemicals already listed in Chapter 2 the following reagents were obtained from the Sigma Chemical Company, St Louis, USA: sodium guanosine 5'-triphosphate (GTP); crystalline uridine; crystalline 5-fluoro-2'-deoxyuridine (5-F-Udr); crystalline 5-bromo-2'-deoxyuridine (5-Br-Udr); crystalline 5-iodo-2'-deoxyuridine (5-I-Udr); sodium uridine 5'-triphosphate (UTP); crystalline cytidine and crystalline 2'-deoxycytidine (Cdr). Crystalline thymine and uracil were purchased from British Drug Houses Ltd., Poole, England.

Radiochemicals

The following radiochemicals were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England: thymidine-(methyl- ^3H), in 10% aqueous-ethanol solution, (^3H -Tdr, 5.0Ci/mmol); thymidine-(methyl- ^3H) 5'-monophosphate, as the ammonium salt in 50% aqueous-ethanol, (^3H -dTMP, 20Ci/mmol); adenosine-5'(γ - ^{32}P)-triphosphate, sodium salt in 50% aqueous-ethanol, (γ - ^{32}P -ATP, 2.9mCi/mol).

Other Chemicals and Reagents

All other reagents used were of Analar grade and were supplied by the Sigma, Merck, BDH, and Malinkrodt chemical companies.

Chromatographic Materials

Whatman microgranular DE 52 (pre-swollen DEAE-cellulose) and Whatman cellulose phosphate P11 were obtained from W. & R. Balston (Modified Cellulose) Ltd., Kent, England. CM Sephadex G50 and Sephadex G25 (medium particle size) were

obtained from Pharmacia Ltd., Uppsala, Sweden. Biogel A1.5 (100-200 mesh) was purchased from Bio-Rad Laboratories, California, USA.

The anion exchangers were prepared and packed according to the method of Himmelhoch (1971).

DE 52 cellulose was suspended in 20mM HCl and degassed using a vacuum pump. Tris-(hydroxymethyl)-aminomethane (Tris) was added until the pH was 8.0. The cellulose was washed several times with 20mM Tris-HCl buffer, (pH 8.0), containing 20% glycerol and 10mM β -mercaptoethanol, and was packed in a vertical column and washed with the same buffer until the pH and conductivity of the effluent were the same as those of the equilibrating buffer.

Phosphocellulose P11 was washed using 0.5N HCl, water, 0.5N NaOH, water, 0.5N HCl, and water. It was then degassed, and washed several times with 10mM imidazole-HCl buffer at pH 6.5, containing 20% glycerol and 10mM β -mercaptoethanol. The cellulose was packed in a vertical column and equilibrated with the same buffer until the pH and conductivity of the effluent were the same as those of the equilibrating buffer.

CM Sephadex C50 (cation exchanger) was swollen in deionised water, washed in 0.5N NaOH, water, 0.5N HCl, water, 0.5N NaOH, and water; degassed, and equilibrated in 10mM potassium phosphate buffer (pH 6.0), containing 20% glycerol and 10mM β -mercaptoethanol. The gel was packed in a vertical column, and washed with the same buffer until the pH and conductivity of the effluent were the same as those of the equilibrating buffer.

Sephadex G25 and Biogel A1.5 gels were pre-swollen in the appropriate equilibrating buffers for the required periods of time, and were packed in vertical columns and equilibrated as described by Reiland, (1971).

Human Tissues

Human Adult Liver was obtained from the Tissue Bank of The South African Institute for Medical Research. The donors were an elderly White female who had died of congestive cardiac failure, and a sixty-three year old White male who had died of a coronary thrombosis. (In the latter case there was no evidence of secondary liver damage).

Human Hepatomas were obtained at autopsy on primary liver carcinoma patients. Most of the studies on hepatoma Tdr kinases were carried out using tissue from a twenty-seven year old male of the Shangaan tribe who had received a single dose (15g) of urea chemotherapy.

Both normal and cancerous human liver tissues were obtained within 6-8 hours of death and were stored frozen at -20°C until used. Normal liver tissue from a healthy young Black male would have served as the most appropriate control in these comparative studies. Obtaining such tissue in under 36 hours after death proved, however, to be a practical impossibility.

METHODS

Initial Assay Procedures

Thymidine Kinase (Tdr kinase)

Tdr kinase was assayed as described in Chapter 2 (page 68).

Thymidylate Kinase (dTMP kinase)

The amount of this enzyme remaining at various stages of the Tdr kinase purification procedure was assayed. The assay conditions for dTMP kinase thus differed from those described in Chapter 2 in that the concentrations of ATP and MgCl_2 used were those which were found to be optimal for Tdr kinase activity. Each assay mixture contained 50mM Tris-HCl, pH 8.0 ; 5mM ATP (adjusted to pH 7 with sodium bicarbonate); 5mM MgCl_2 ; 50 μM ^3H -dTMP (approximately 100mCi/mmol); and enzyme preparation,

in a final volume of 200 μ l. The assay procedure was as described in Chapter 2 (page 70).

Thymidylate Phosphatase (dTMPase)

Activities of specific or non-specific enzymes capable of dephosphorylating dTMP were estimated using an assay mixture containing: 50mM Tris-HCl, pH 8.0; 5mM MgCl₂; 50 μ M ³H-dTMP (approximately 100mCi/mmol); and enzyme preparation, in a final volume of 200 μ l. Incubations were performed at 37°C for 0 and 20 minutes, and assays were terminated by heating tubes in a boiling water bath for 5 minutes. Denatured protein was spun down, and a 25 μ l aliquot of each assay mixture supernatant was spotted onto a 25 x 2cm strip of Whatman DE 81 cellulose paper. ³H-dTMP and ³H-Tdr were separated by ascending chromatography using 0.1M ammonium formate-1N formic acid as the solvent, as described on page 70 . Marker spots for Tdr and dTMP were located under ultra-violet light of 254nm wavelength. Segments corresponding to the positions of Tdr and dTMP were cut from each dried strip and counted in a scintillation cocktail as described in Chapter 2 (page 75). The counts corresponding to the dTMP spots in the zero-time blanks were used as the 'total counts' for each enzyme preparation assayed. The assays for dTMP kinase and dTMPase were subsequently modified as described on page 128 .

Adenosine Triphosphatase (ATPase)

Initially the colorimetric method described by Lowry (1957) was used. The method measures liberated phosphate by reaction with molybdate at pH 4, using ascorbic acid as a reducing agent. This method was found to be insensitive and it was therefore decided to use an isotopic assay for ATPase determinations.

Each assay mixture contained: 50mM Tris-HCl, pH 8.0; 5mM MgCl_2 ; 5mM γ - ^{32}P -ATP (containing 0.5 to 3.0mCi/mmol); and suitable dilutions of enzyme preparation, in a final volume of 200 μl . Incubations were performed at 37°C for 0 and 20 minutes. Assays were terminated by the addition of one volume of ice-cold 10% trichloroacetic acid. Denatured protein was spun down and the ^{32}P -ATP and ^{32}P -inorganic phosphate in 25 μl aliquots of each assay mixture supernatant were separated chromatographically, in the same manner as for the dTMPase assay (page 124). Inorganic phosphate was found to have an R_f value of 0.8, while that of ATP was 0.2. Segments corresponding to these spots were cut out of each DE 81 strip and counted in 10ml of scintillation cocktail as described in Chapter 2 (page 73), using optimum gain and window settings for ^{32}P . The counts corresponding to the ATP spots in the zero-time blanks were used as the 'total counts' for each enzyme preparation assayed.

Thymidine Analogue Studies

The ability of various thymidine analogues to serve as substrates for Tdr kinase was investigated using a preparation from human normal liver. Kinase activity was monitored by the formation of ^{32}P -labelled nucleoside or deoxynucleoside monophosphates when the test substrate was incubated in the presence of enzyme preparation and ^{32}P -ATP. Assay mixtures contained 50mM Tris-HCl buffer, pH 8.0; 100 μM ^{32}P -ATP (containing 20mCi/mmol); 0.4mM MgCl_2 ; 20 μM test substrate; and suitably diluted enzyme preparation, in an assay volume of 200 μl . Assays were initiated by addition of ^{32}P -ATP. A 100 μl zero-time aliquot was removed immediately and the remaining assay mixture was incubated at 37°C for 20 minutes. Assays were terminated by the addition of one volume of cold 10% trichloroacetic acid.

Denatured protein was spun down, and 25µl of each protein-free supernatant was spotted onto a 2 x 25cm strip of DE 81 ion-exchange paper. Nucleotides were separated by ascending chromatography using the 1N formic acid-0.1N ammonium formate system described on page 70. Appropriate marker spots were used to locate the ^{32}P -labelled nucleoside or deoxynucleoside products. The counts corresponding to the ATP spots in the zero-time blanks were used as the 'total counts' for each enzyme preparation assayed. Samples were counted as described on page 75, using optimum gain and window settings for ^{32}P .

Protein Determination

Proteins were determined by the method of Lowry *et al.* (1951) as described on page 76, using bovine serum albumin as a reference protein.

Units of Enzyme Activity

All enzyme activities are expressed as Units of activity per assay or per gram protein, one Unit being defined as the amount of enzyme catalysing the formation of 1nmole of product per minute at 37°C.

Methods Adopted to Reduce ATPase Activity in Thymidine Kinase Assays

Since 33 000 x g supernatants of normal liver and hepatoma were found to have very high ATPase activity, attempts were made to reduce this contamination. The results of these investigations are summarised in Table 12. It can be seen that the presence of 20% glycerol in the homogenising buffer (50mM Tris-glycine-cacodylate buffer, pH 6.5, containing 10mM β-mercaptoethanol) produced greater ATPase contamination in an extract of Tdr kinase from human normal liver. The absence of glycerol at the initial homogenisation stage did not adversely affect Tdr kinase stability.

Use of a Waring blender instead of a hand-operated Potter homo-

TABLE 12

MODIFICATIONS TO REDUCE ATPase ACTIVITY IN THE DEVELOPMENT OF THE PARTIAL
PURIFICATION PROCEDURE FOR THYMINE KINASE FROM HUMAN NORMAL AND CANCEROUS LIVER

Modification	Homogeniser	Centrifugation (x g)	Assay buffer (pH)	Tdr Kinase Activity ²	ATPase Activity ²	ATPase Tdr Kinase
Presence of 20% glycerol in IM ¹	Waring blender	105 000	Tris-glycine- cacodylate (6.5)	0.019	11.8	632
Absence of 20% glycerol in IM	Waring blender	105 000		0.021	7.4	556
Homogenisation	Potter	105 000	Tris-HCl (8.0)	0.022	10.2	469
	Waring blender	105 000		0.020	10.9	552
Centrifugation (60 min)	Potter	35 000	Tris-HCl (8.0)	0.022	15.8	735
	Potter	105 000		0.022	10.2	469
Assay buffer pH	Waring blender	105 000	Tris-HCl (8.0)	0.020	10.9	552
	Waring blender	105 000	Tris-glycine- cacodylate (6.5)	0.021	7.4	357
	Waring blender	105 000	Imidazole-HCl (6.5)	0.018	5.8	322
Inhibitors of ATPase						
NIL	Waring blender	105 000	Imidazole-HCl (6.5)	0.018	5.8	322
37.5mM sodium fluoride	Waring blender	105 000	Imidazole-HCl (6.5)	0.020	4.2	213
75mM sodium arsenate	Waring blender	105 000	Imidazole-HCl (6.5)	0.012	5.4	455
50mM ouabain	Waring blender	105 000	Imidazole-HCl (6.5)	0.021	5.9	289

1. IM = homogenising medium, which contained 50mM Tris-HCl, pH 7.5, 10mM β -mercaptoethanol and 250mM sucrose.

2. Enzyme activities are expressed as units/100 μ l Tdr kinase preparation.

geniser to homogenise human normal liver produced a slight increase in ATPase contamination of the Tdr kinase preparations (Table 12).

It was, however, decided to use the Waring blender as this afforded a more rapid, reproducible and convenient means of homogenising the large quantities of tissue required for the purification procedure.

From Table 12 it is clear that centrifugation at $105\ 000 \times g$ did not alter the total Tdr kinase activity extracted when compared with centrifugation at $35\ 000 \times g$, but reduced ATPase contamination by some 35%.

The assay conditions for Tdr kinase were also modified. As can be seen from Table 12, ATPase activity was 550-times higher than that of Tdr kinase when Tris-HCl buffer at pH 8.0 was used. By using a lower pH assay buffer, imidazole-HCl, pH 6.5, the activity of the contaminating ATPase was reduced by 40% without any effect on Tdr kinase activity.

ATPase activity could be further reduced by assaying in the presence of 37.5mM sodium fluoride, without any adverse effect on Tdr kinase activity (Table 12). Sodium arsenate (75mM) and ouabain (50 μ M) did not inhibit the ATPase activity.

In order to reduce the effect of ATPase contamination of Tdr kinase preparations: (1) $105\ 000 \times g$ supernatants were prepared, allowing a 40% absolute reduction in ATPase activity and (2) assay conditions were chosen so as to be less favourable for ATPase, without affecting Tdr kinase activity. The assay procedures for Tdr kinase, dTMP kinase, and dTMP phosphatase (page 123) were accordingly modified by the use of 50mM imidazole-HCl buffer, pH 6.5, and the addition of 37.5mM sodium fluoride to all assay mixtures.

The assay procedure described on page 124 for ATPase was similarly modified.

Partial Purification of Thymidine Kinases from Human Adult Liver
and Hepatoma - Preliminary Studies

Ammonium Sulphate Fractionation

10g of human normal liver was homogenised in four volumes of 50mM Tris-HCl, pH 7.5, containing 250mM sucrose and 10mM β -mercaptoethanol, using a Potter-Elvehjem homogeniser. The homogenate was centrifuged for 30 minutes at 38 000 x g in a refrigerated MSE 18 centrifuge. Solid ammonium sulphate was added slowly, with gentle stirring, to the supernatant at 4°C until 25% saturation was reached. A concentration of 70 grams per 100ml was accepted as being equivalent to 100% saturation at 4°C. The solution was stirred for a further 10 minutes and then centrifuged at 38 000 x g. The ammonium sulphate concentration in the supernatant was increased by increments of 5%, until 60% saturation was reached. The protein precipitate obtained after each increase in ammonium sulphate saturation was redissolved in 50mM Tris-HCl, pH 7.5, containing 250mM sucrose and 10mM β -mercaptoethanol, and assayed for Tdr kinase activity.

Tdr kinase activity from human normal liver tissue was precipitated at ammonium sulphate concentrations of between 35 and 60%. This fractionation step resulted in a 50% loss of Tdr kinase activity which could not be recovered by desalting the enzyme preparation. In addition ammonium sulphate fractionation did not reduce the ATPase contamination and it was therefore decided not to include this step in the Tdr kinase purification procedure.

Preparation of a High-speed Supernatant Fraction

As has been described above (page 128), preparation of a 105 000 x g supernatant significantly reduced the levels of contaminating ATPase activity.

Stabilisation of Thymidine Kinase Activity

Both β -mercaptoethanol and 20% glycerol were necessary to stabilise Tdr kinases during the purification procedures. β -mercaptoethanol (10mM) was included in all buffers used in the purification procedure. Glycerol could not be added to the homogenising buffer, since it led to increased ATPase contamination of the Tdr kinase preparations (page 126). Glycerol was therefore added to the high-speed supernatant preparations to achieve a 20% (v/v) concentration, and 20% glycerol was present in all buffers used in subsequent purification steps.

Final Purification Procedure for Purification of Thymidine Kinases from Human Adult Liver and Hepatoma

All purification steps were carried out at 0-4°C unless otherwise indicated. Where necessary enzyme preparations were stored at -20°C between purification steps. The procedure used in the Tdr kinase purifications is summarised in Figure 11.

Extraction of Thymidine Kinase Activity

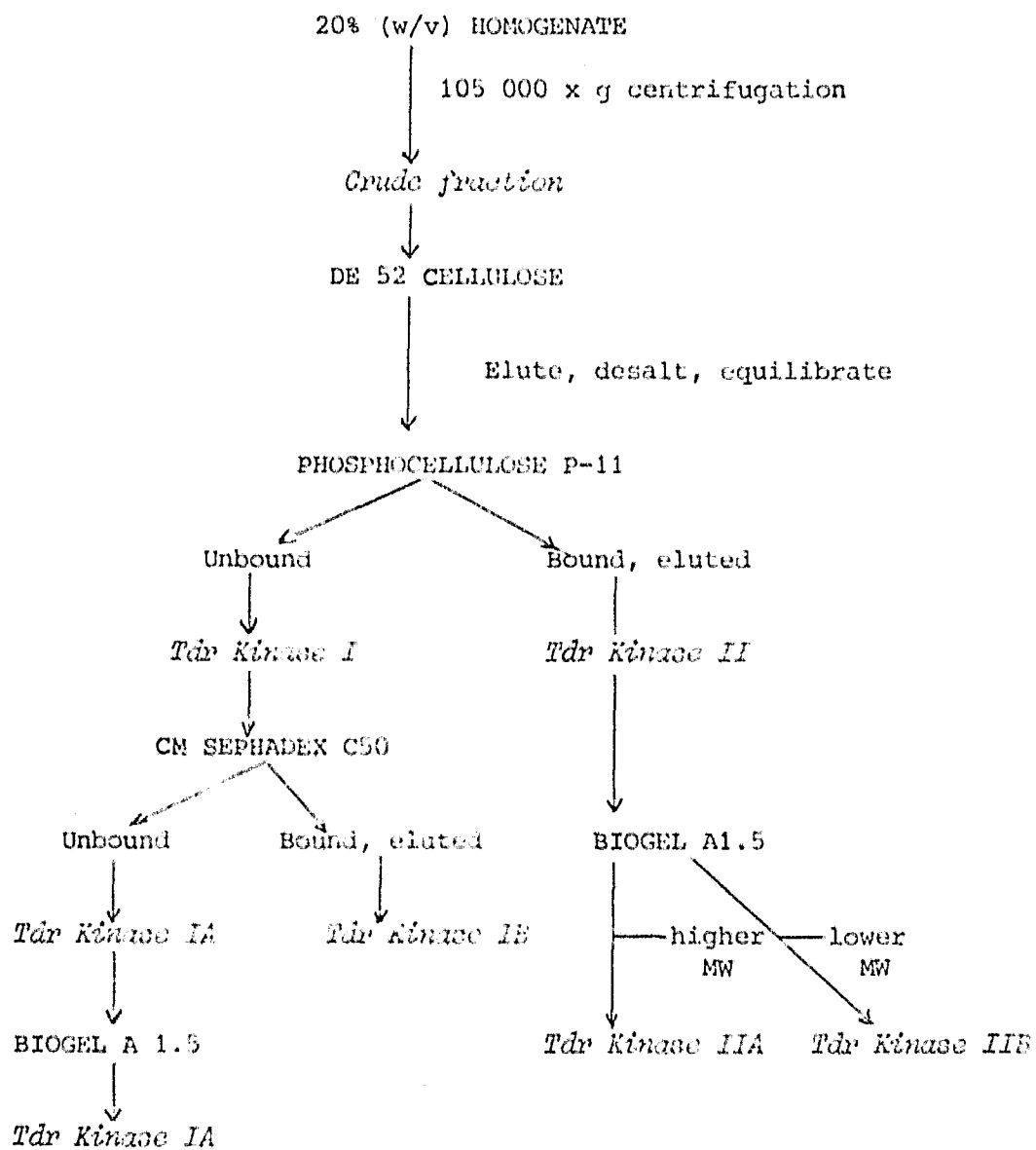
Liver or hepatoma tissues were thawed, and dissected free of cirrhotic or necrotic tissue. Approximately 200g of the tissue was cut into small pieces and homogenised in a Waring blender with four volumes of 20mM Tris-HCl buffer, pH 8.0, containing 250mM sucrose and 20mM β -mercaptoethanol. Three homogenising periods, each of 45 seconds, were used. The homogenate was centrifuged for 90 minutes at 105 000 x g, using a Beckman L5-65 ultracentrifuge and a SW 27 rotor. The supernatant fraction was filtered through glass wool, and brought to 20% saturation with glycerol. This is referred to as the crude fraction.

DEAE-cellulose Chromatography

The crude fraction was applied to a DE 52 column (20 x 6cm) equilibrated with 20mM Tris-HCl, pH 8.0, containing 20% glycerol and

FIGURE 11.

FLOW DIAGRAM OF PURIFICATION STAGES USED IN THE PARTIAL
PURIFICATION OF THYMIDINE KINASES FROM HUMAN NORMAL LIVER
AND HEPATOMA



MW - molecular weight.

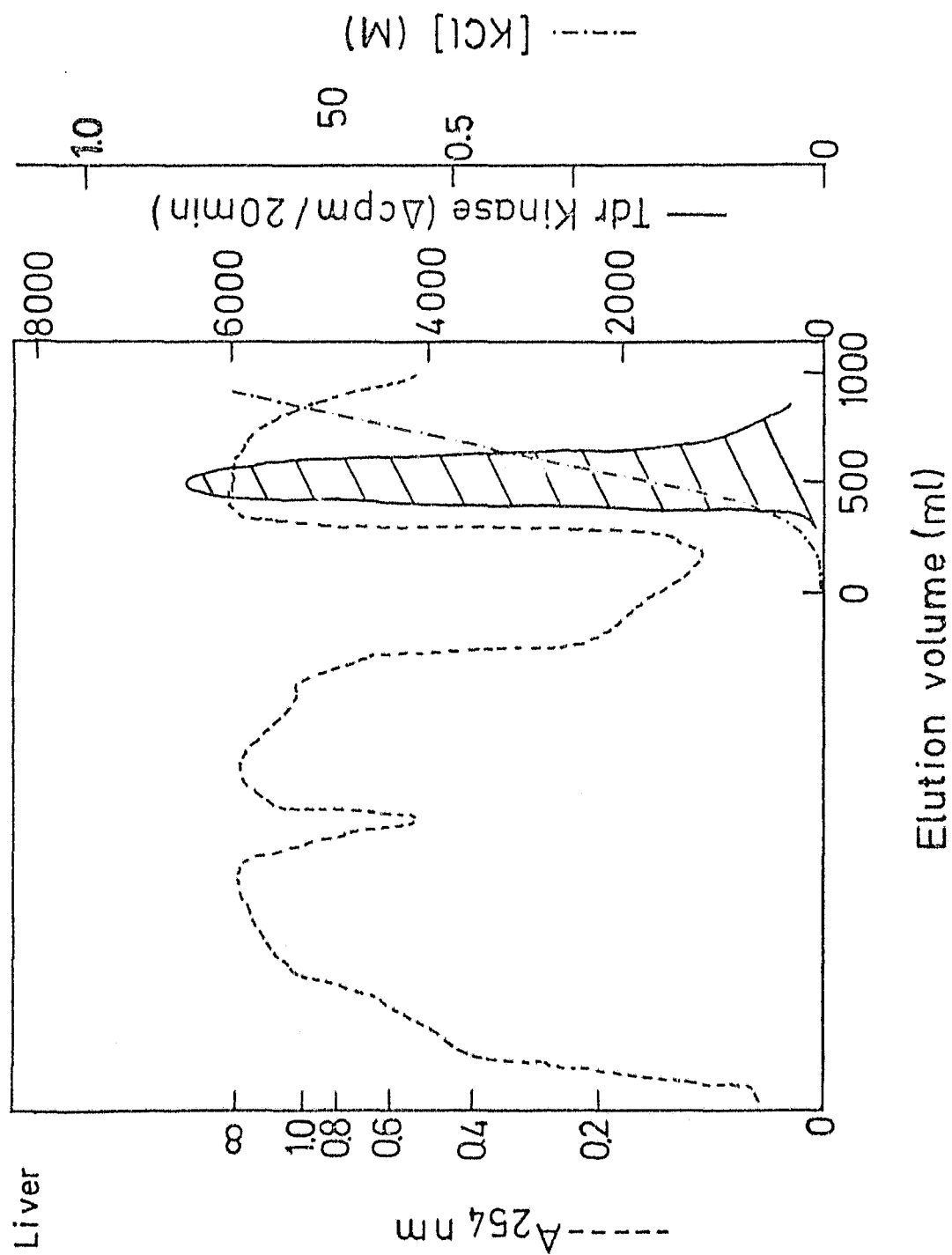
10mM β -mercaptoethanol. The column was washed with the equilibrating buffer until all unbound protein, which contained no Tdr kinase activity, had been eluted. The protein bound to the column was then eluted with one litre of a linear gradient of 0 to 1M KCl in equilibrating buffer. Fractions of 6.5ml were collected and assayed for Tdr kinase activity. The elution profiles for human liver and hepatoma Tdr kinases are shown in Figures 12 and 13 respectively. The active fractions were pooled, and concentrated using an Amicon ultrafiltration cell with a PM 10 membrane.

The concentrated preparation was desalted on a Sephadex G25 column previously equilibrated with 10mM imidazole-HCl buffer, pH 6.5, containing 20% glycerol and 10mM β -mercaptoethanol. Protein was eluted using the same buffer. The pH and conductivity of the eluted protein were checked to ensure that they corresponded to those of the imidazole-HCl buffer.

Phosphocellulose P11 Chromatography

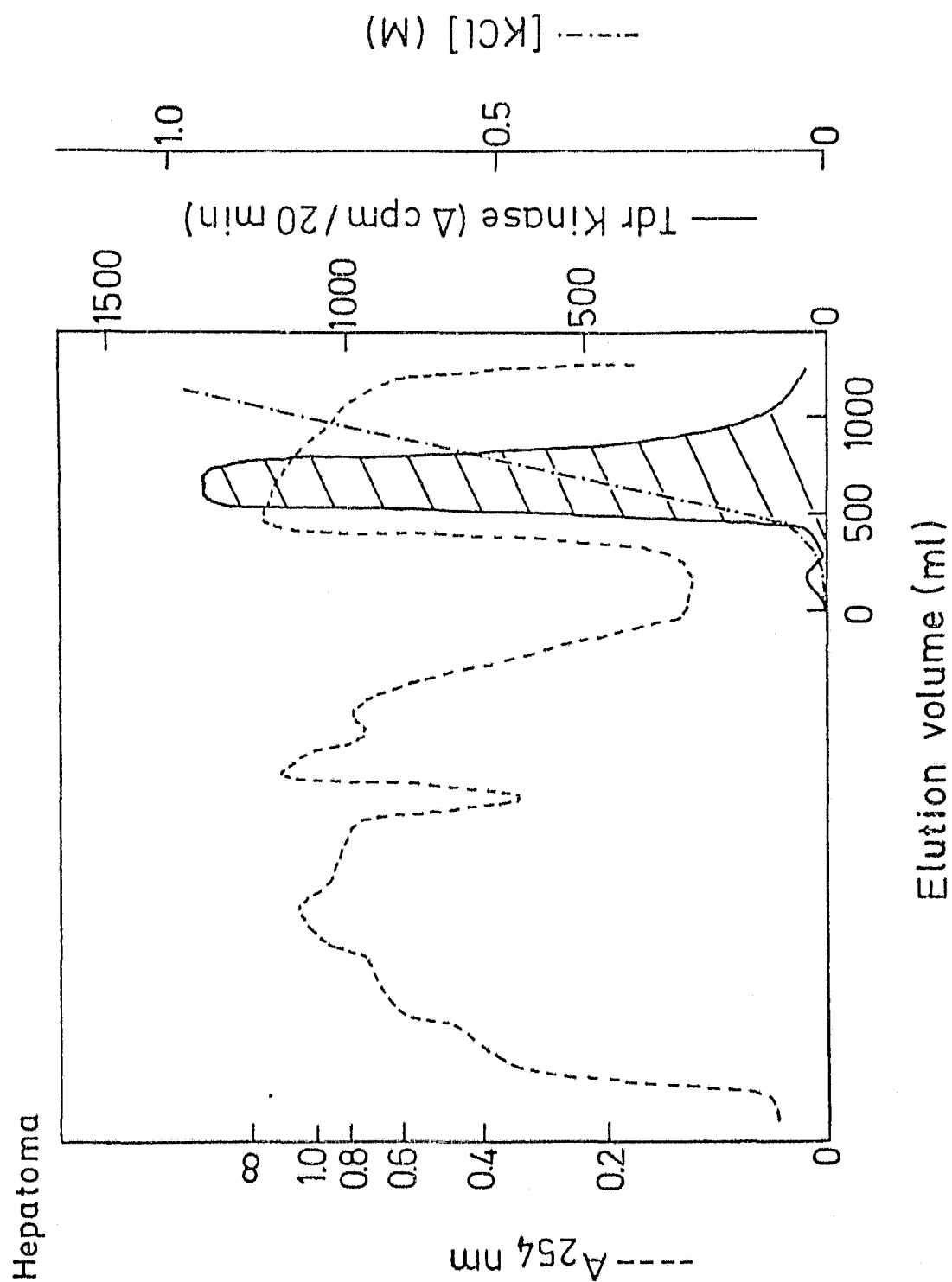
The enzyme preparation was applied to a phosphocellulose P11 column (6 x 6cm) equilibrated with 10mM imidazole buffer, pH 6.5, containing 20% glycerol and 10mM β -mercaptoethanol. The column was washed with the same buffer to remove unbound protein. Fractions of 6.5ml were collected, and assayed for Tdr kinase activity. Active fractions were pooled and concentrated in an Amicon ultrafiltration cell using a PM 10 membrane, and designated Tdr K I. Protein bound to the column was eluted with 500ml of a linear 0 to 2M KCl gradient in 100mM imidazole-HCl buffer, pH 6.5, containing 20% glycerol and 10mM β -mercaptoethanol. Fractions of 6.5ml were collected and assayed for Tdr kinase activity. The elution profiles for human liver and hepatoma Tdr kinases are shown in Figure 14. Active fractions were pooled, concentrated in an Amicon ultrafiltration cell using a PM 10 membrane, and designated Tdr K II.

FIGURE 12
THE ELUTION PROFILE FOR HUMAN LIVER THYMIDINE KINASE ON DE 52 CELLULOSE



The shaded area represents Tdr kinase activity. The column dimensions were 6 x 20cm, and Tdr kinase activity was eluted with a 0-1.0M KCl linear gradient in 20mM Tris-HCl buffer, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol.

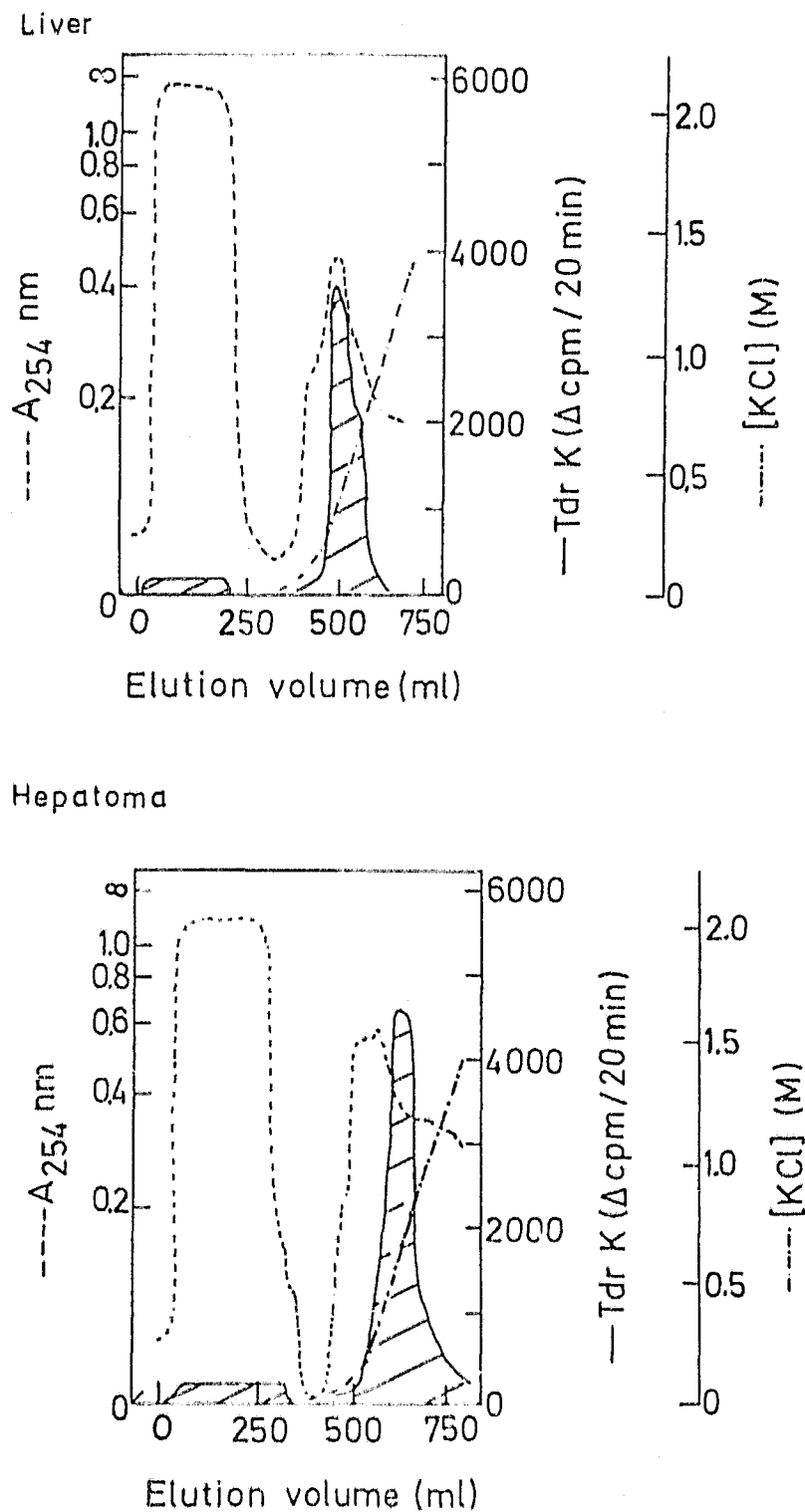
FIGURE 13
THE ELUTION PROFILE FOR HUMAN HEPATOMA THYMIDINE KINASE ON DE 52 CELLULOSE



The shaded area represents Tdr kinase activity. The dimensions of the column were 6 x 20cm, and Tdr kinase activity was eluted using a 0-1.0M KCl linear gradient in 20mM Tris-HCl buffer, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol.

FIGURE 14

THE ELUTION PROFILES FOR HUMAN LIVER AND HEPATOMA
THYMIDINE KINASES ON PHOSPHOCELLULOSE P11 COLUMNS



The shaded areas represent Tdr kinase activity. The dimensions of the columns were 6 x 6cm. Unbound Tdr kinase activity was washed off using 10mM imidazole-HCl buffer, pH 6.5, containing 20% glycerol and 10mM β-mercaptoethanol. Bound Tdr kinase activity was eluted using a 0-2M KCl gradient in 100mM imidazole-HCl buffer, pH 6.5, containing 20% glycerol and 10mM β-mercaptoethanol.

Further Fractionation of Tdr K II Using Gel Filtration
on Biogel A1.5

The Tdr K II preparation was applied to a Biogel A1.5 column (66x2cm) which had been equilibrated with 20mM Tris-HCl buffer, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol. Protein was eluted using the same buffer. Fractions of 3ml were collected and assayed for Tdr kinase activity. The elution profiles for human liver and hepatoma Tdr kinases are shown in Figures 15 and 16 respectively. As two peaks of activity were obtained these were pooled separately. The peak eluting first (ie. of higher molecular weight) was designated Tdr K IIA, and the second was designated Tdr K IIB.

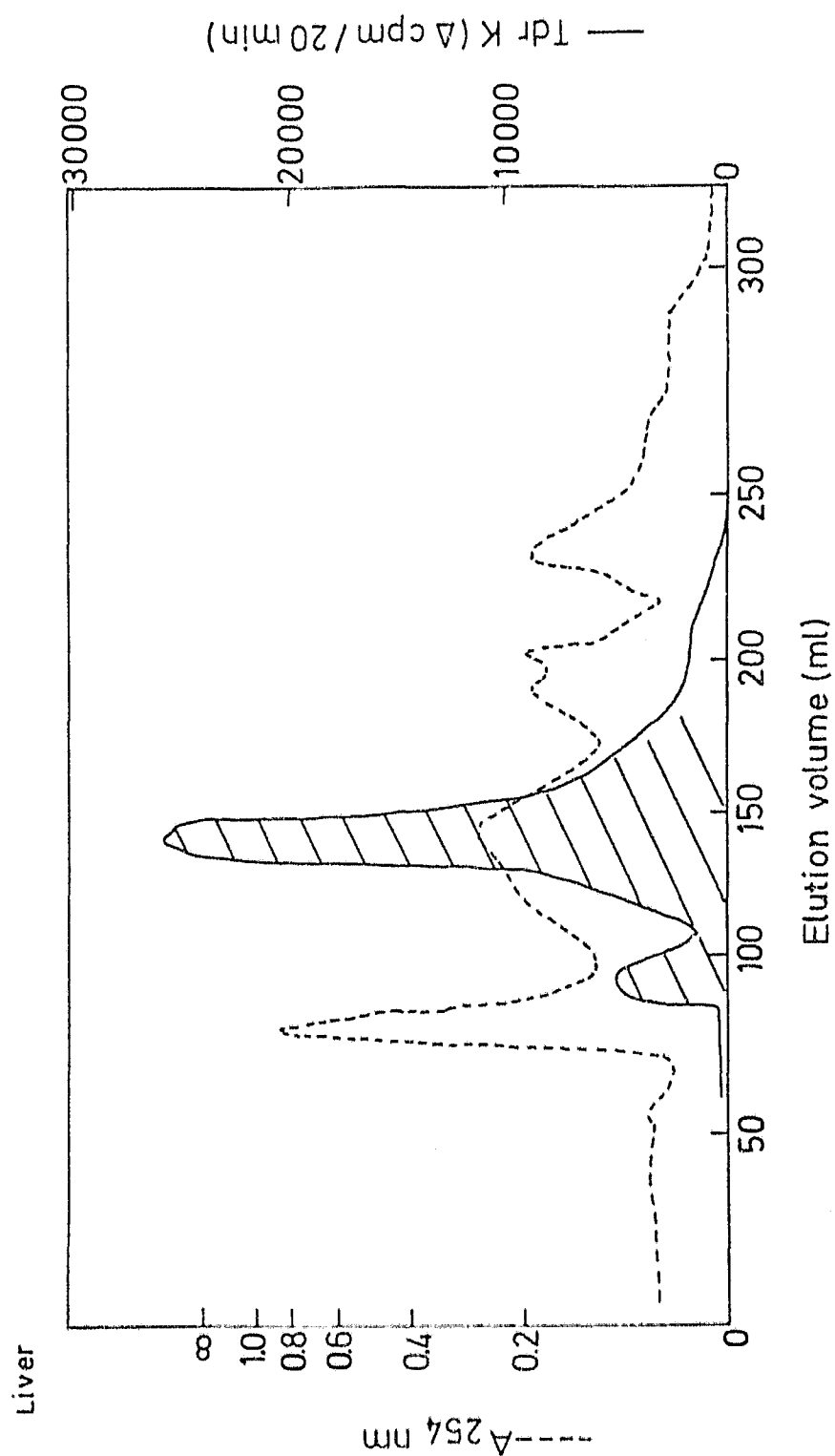
Further Purification of Tdr K I Using CM-Sephadex C50 Chromatography

The Tdr K I preparation was applied to a Sephadex G25 column equilibrated with 10mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 10mM β -mercaptoethanol. The protein was eluted with the same buffer, and the pH and conductivity of the eluted protein were measured to ensure that they were similar to those of the phosphate buffer.

This preparation was then applied to a CM-Sephadex C50 column (14 x 4cm) equilibrated with 10mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 10mM β -mercaptoethanol. The column was washed with this buffer to remove unbound protein. Fractions of 6.5ml were collected and assayed for Tdr kinase activity. The unbound active fractions were pooled, concentrated using an Amicon ultrafiltration cell with a PM 10 membrane, and designated Tdr K IA.

Bound protein was then eluted with 500ml of a linear gradient of 0 to 1M KCl in 10mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 10mM β -mercaptoethanol. Fractions of 6.5ml were collected and assayed

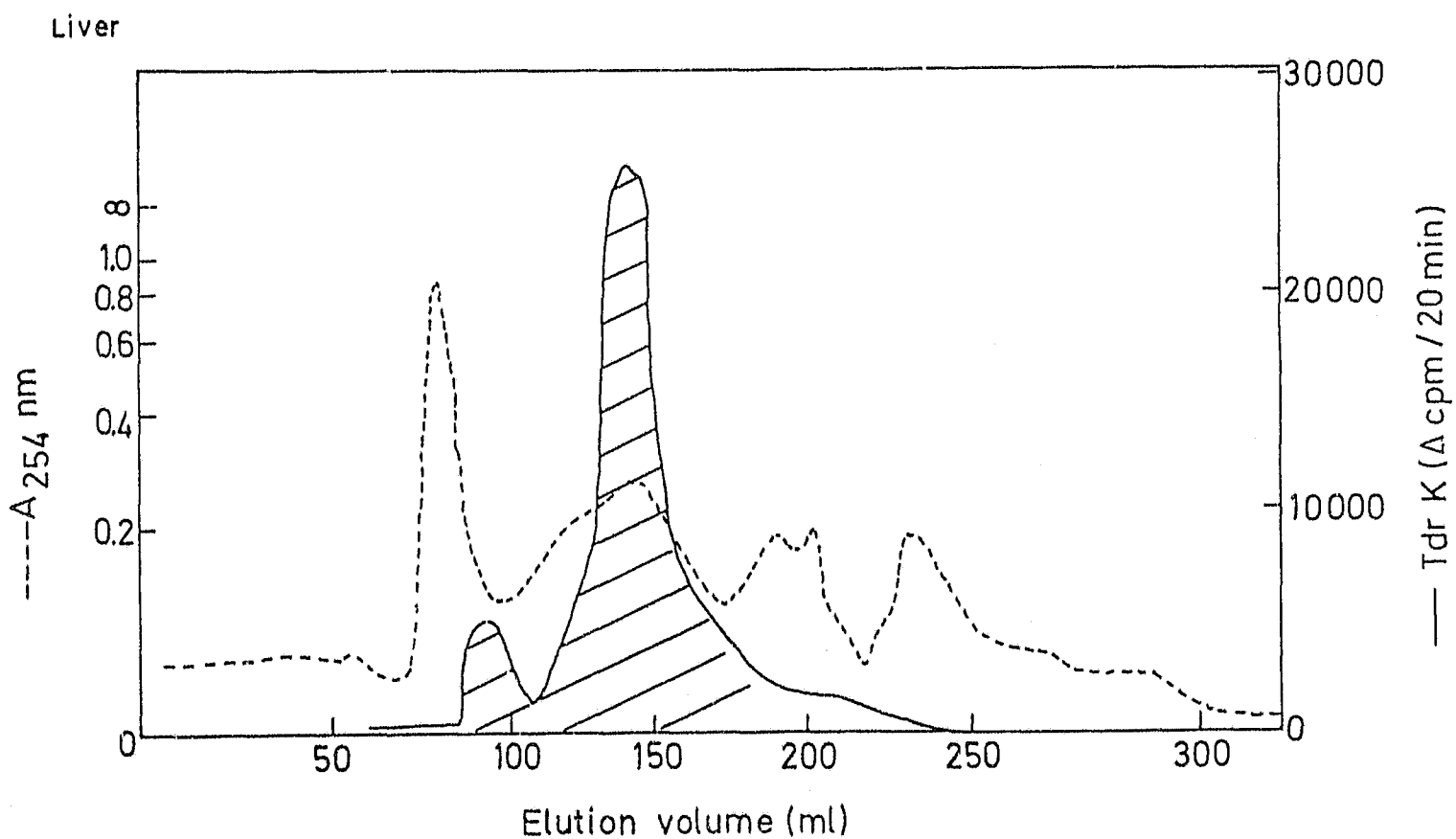
FIGURE 15
 THE ELUTION PROFILE FOR THE HUMAN LIVER THYMIDINE
 KINASE II PREPARATION ON BIOGEL A1.5



The shaded areas represent Tdr kinase activity. The dimensions of the column were 2 x 66cm, and the eluting buffer was 20mM Tris-HCl, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol.

FIGURE 15

THE ELUTION PROFILE FOR THE HUMAN LIVER THYMIDINE
KINASE II PREPARATION ON BIOGEL A1.5



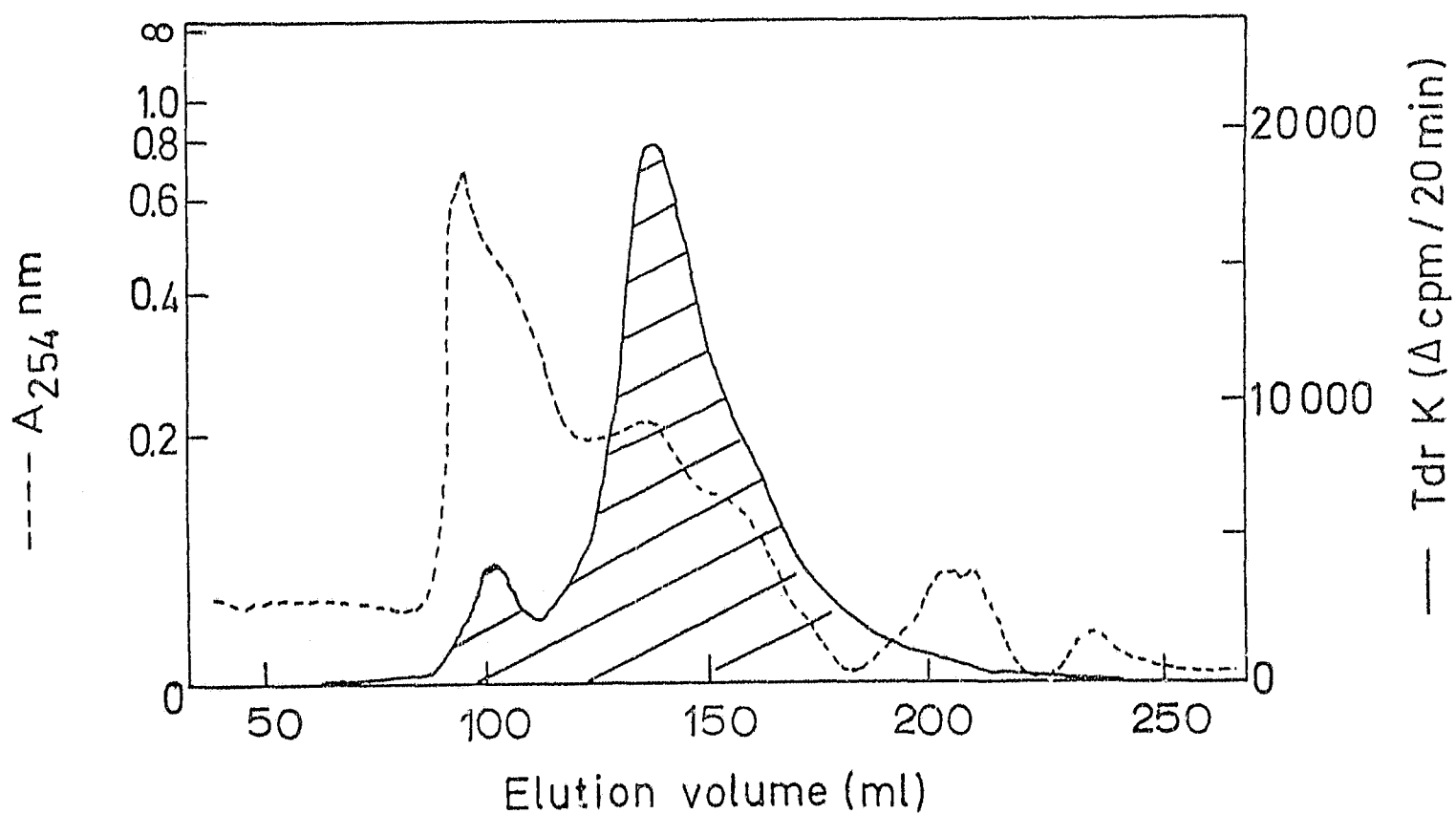
The shaded areas represent Tdr kinase activity.

The dimensions of the column were 2 x 66cm, and the eluting buffer was 20mM Tris-HCl, pH 8.0, containing 20% glycerol and 10mM β-mercaptoethanol.

FIGURE 16

THE ELUTION PROFILE FOR THE HUMAN HEPATOMA THYMIDINE
KINASE II PREPARATION ON BIOGEL A1.5

Hepatoma



The shaded areas represent Tdr kinase activity.

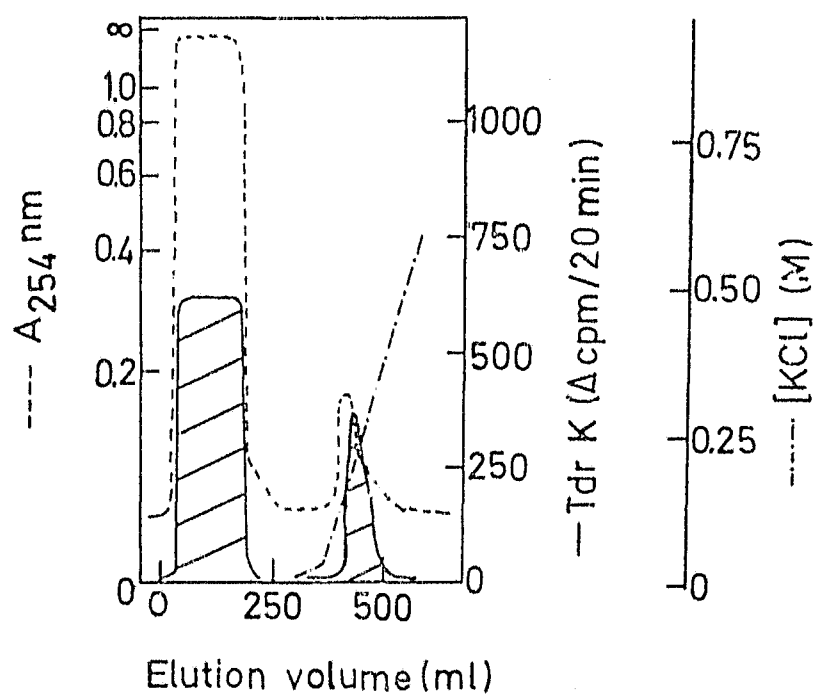
The dimensions of the column were 2 x 66cm, and the eluting buffer was 20mM Tris-HCl, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol.

FIGURE 17

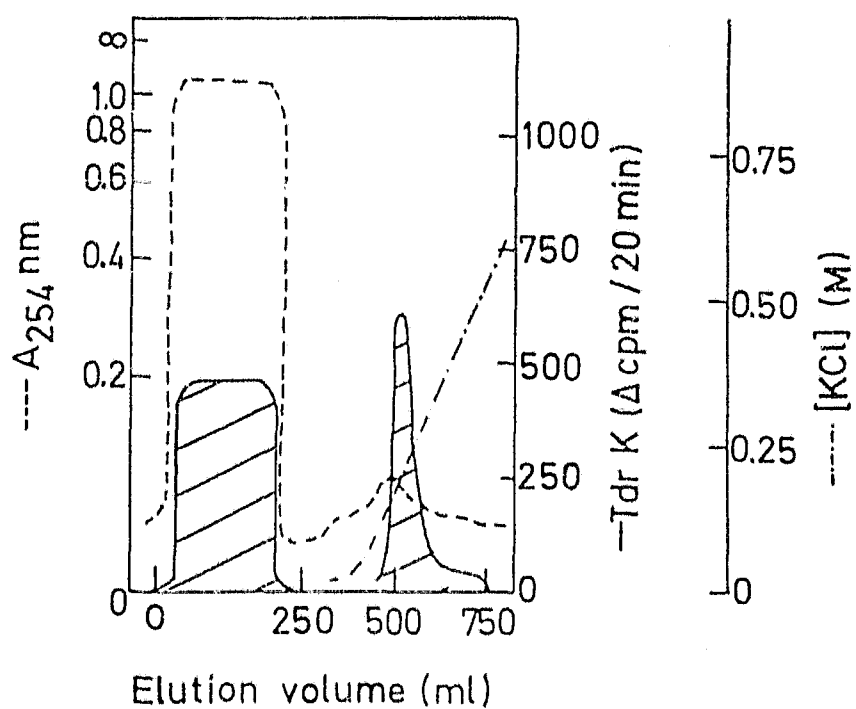
THE ELUTION PROFILES FOR HUMAN LIVER AND HEPATOMA

THYMIDINE KINASES ON CM-SEPHADEX C50 COLUMNS

Liver



Hepatoma



The shaded areas represent Tdr kinase activities.
 The dimensions of the columns were 4 x 4cm. Bound Tdr kinase activity was eluted using a 0-1M KCl linear gradient in 10mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 10mM β-mercaptoethanol.

for Tdr kinase activity. Active fractions were pooled, concentrated in an Amicon ultrafiltration cell using a PM 10 membrane, and designated Tdr K IB. The elution profiles for human liver and hepatoma Tdr kinases are shown in Figure 17.

Further Purification of Tdr K IA Using Gel Filtration on Biogel A1.5

The Tdr K IA preparation was applied to a Biogel A1.5 column which had been equilibrated with 20mM Tris-HCl buffer, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol. Fractions of 3ml each were collected and assayed for Tdr kinase activity. The elution profiles for human liver and hepatoma Tdr kinases are shown in Figure 18. Active fractions were pooled, and concentrated using an Amicon ultrafiltration cell with a PM 10 membrane.

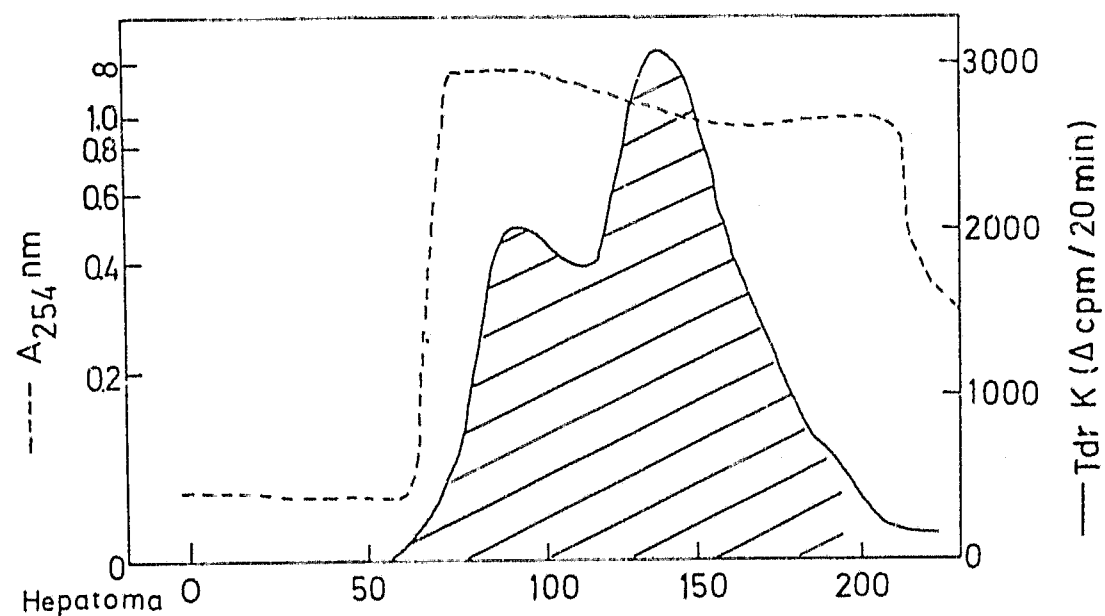
Estimation of the Molecular Weight of Thymidine Kinases Using a Calibrated Biogel A1.5 Column

A Biogel A1.5 column was packed and equilibrated as described on page 122, using 20mM Tris-HCl buffer (pH 8.0), containing 10mM β -mercaptoethanol, 20% glycerol and 100mM KCl. The dimensions of the column were 2 x 56cm. The column was calibrated using 2ml samples (containing between 2 and 50mg/ml of protein) of the following proteins of known molecular weight: glutamate dehydrogenase (300 000); pyruvate kinase (237 000); aldolase (180 000); hexokinase (99 000); enolase (82 000); carboxypeptidase B (35 000); and cytochrome C (12 750). Samples of Tdr kinases IA, IB, IIA, and IIB from human normal and cancerous liver tissue were then eluted from the column and, in each case, the volume required to elute the protein fraction giving the highest absorbance at 254nm was noted. Using the modified method of Andrews (1967) the elution volume of each of the standard protein preparations was plotted against the logarithm of its

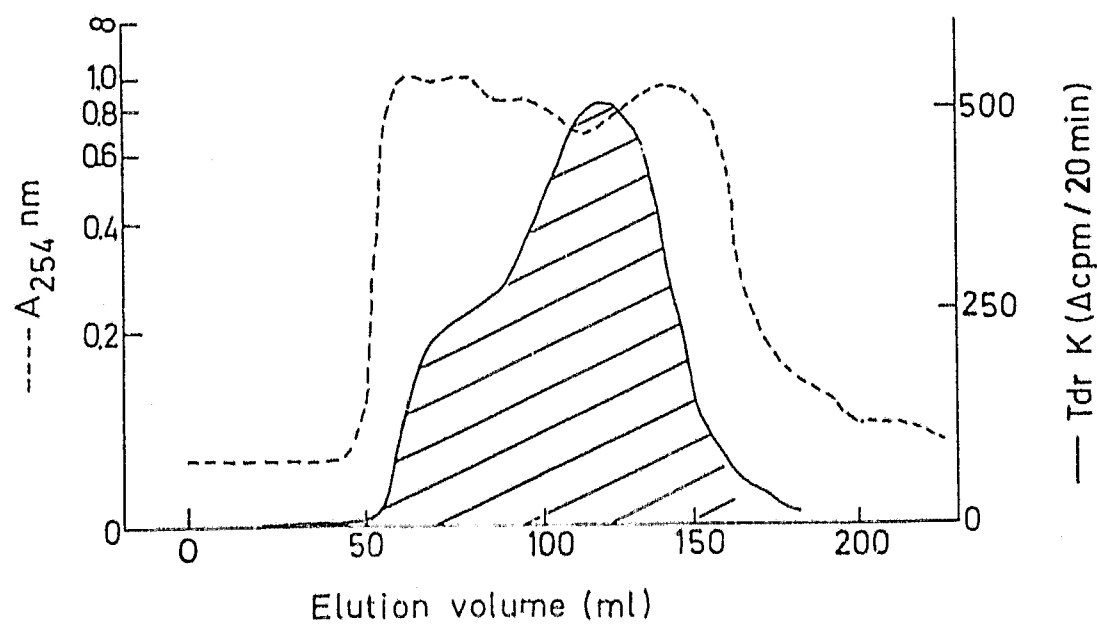
FIGURE 18

THE ELUTION PROFILES FOR HUMAN LIVER AND HEPATOMA
 THYMIDINE KINASE IA PREPARATIONS ON BIOGEL A1.5

Liver



Hepatoma



The shaded areas correspond to Tdr kinase activity.
 The dimensions of the column were 2 x 66cm, and the eluting buffer was
 20mM Tris-HCl, pH 8.0, containing 20% glycerol and 10mM β -mercaptoethanol.

molecular weight, and the molecular weights for the Tdr kinase preparations were read off the resulting graph.

Determination of Kinetic Parameters

Kinetic data were plotted as reciprocal velocity versus reciprocal substrate concentrations at a fixed concentration of the second substrate. Data giving linear plots were fitted to the equation

$$v = \frac{V \cdot A}{K + A}$$

where v is the observed velocity, V the maximum velocity, A the Tdr or ATP concentration, and K the Michaelis constant for Tdr or ATP. Data were analysed using the computer programme of Cleland (1963), which is based on a least-squares fit of the data points to the equation and gives values for the constants and their standard errors.

RESULTS

PURIFICATION AND CHARACTERISATION OF THYMIDINE KINASES FROM HUMAN

NORMAL LIVER AND HEPATOMA

Purification of Human Liver and Hepatoma Thymidine Kinases

The results obtained using the purification procedure described for human normal liver and hepatoma Tdr kinases are summarised in Tables 13 and 14 respectively. The elution profiles obtained on ion exchange and Biogel chromatography are shown in the Methods section (Figures 12 to 18).

It can be seen from Table 13 that the partial purification procedure for human liver Tdr kinase gave four fractions of Tdr kinase activity: Tdr K's IA, IB, IIA, and IIB. The purification factors of these Tdr kinase preparations, expressed as the ratio of the specific activity of each Tdr kinase preparation relative to the specific activity of Tdr kinase in the supernatant fraction, were 2.8, 6.4, 10.6, and 146 respectively. These represent minimum purification factors for each of the Tdr kinase preparations, since the activity of each is expressed relative to the activity of the four enzyme forms present in the crude fraction. The overall yields for the four enzyme forms were 5%, 1%, 2%, and 22% respectively.

The partial purification procedure for Tdr kinase from human hepatoma tissue again gave four forms of the enzyme: Tdr K's IA, IB, IIA, and IIB (Table 14). The minimum purification factors obtained for these forms were 2.1, 26.1, 31, and 48.8 respectively, while the yields were 7%, 1%, 4%, and 32%.

The relative proportions of the four forms of Tdr kinase from both normal liver and hepatoma are shown in Table 15, from which it is clear that the distribution of the Tdr kinase forms was similar in

TABLE 13

PARTIAL PURIFICATION OF HUMAN NORMAL LIVER THYMIDINE KINASES

Purification Stage	Total Activity (mUnits)	Total Protein (mg)	Specific Activity mUnits/g protein	Yield (%)	Overall Purification Factor*
105 000 x g Supernatant	256	18 100	14.1	100	1.0
DE 52 Cellulose Chromatography	144	3 290	43.8	56	3.1
<u>Phosphocellulose P 11 Chromatography</u>					
Unadsorbed (Tdr K I)	24	2 660	9.2	9	0.65
Adsorbed (Tdr K II)	75	190	395	29	28
<u>CM Sephadex Chromatography of Tdr K I</u>					
Unadsorbed (Tdr K IA)	25	2 210	11.3	10	0.8
Adsorbed (Tdr K IB)	2.7	30	90	1	6.4
Biogel A 1.5 Gel Filtration of Tdr K IA	12.9	330	39.1	5	2.8
Biogel A 1.5 Gel Filtration of Tdr K II					
(Tdr K IIA)	4.5	30	150	2	10.6
(Tdr K IIB)	56	27	2 074	22	146

* The overall purification factor compares the specific activity of the Tdr Kinase fraction or fractions at each stage of the purification procedure with the specific activity of the mixture of Tdr kinases in the crude fraction. This therefore represents a minimum purification factor for each of the Tdr kinase forms.

TABLE 13
PARTIAL PURIFICATION OF HUMAN NORMAL LIVER THYMIDINE KINASES

Purification Stage	Total Activity (mUnits)	Total Protein (mg)	Specific Activity mUnits/g protein	Yield (%)	Overall Purification Factor*
105 000 x g Supernatant	256	18 100	14.1	100	1.0
DE 52 Cellulose Chromatography	144	3 290	43.8	56	3.1
Phosphocellulose P 11 Chromatography					
Unadsorbed (Tdr K I)	24	2 660	9.2	9	0.65
Adsorbed (Tdr K II)	75	190	395	29	28
CM Sephadex Chromatography of Tdr K I					
Unadsorbed (Tdr K IA)	25	2 210	11.3	10	0.8
Adsorbed (Tdr K IB)	2.7	30	90	1	6.4
Biogel A 1.5 Gel Filtration of Tdr K IA	12.9	330	39.1	5	2.8
Biogel A 1.5 Gel Filtration of Tdr K II (Tdr K IIA) (Tdr K IIB)	4.5 56	30 27	150 2 074	2 22	10.6 146

* The overall purification factor compares the specific activity of the Tdr kinase fraction or fractions at each stage of the purification procedure with the specific activity of the mixture of Tdr kinases in the crude fraction. This therefore represents a minimum purification factor for each of the Tdr kinase forms.

TABLE 14

PARTIAL PURIFICATION OF HUMAN HEPATOMA THYMIDINE KINASES

Purification Stage	Total Activity (mUnits)	Total Protein (mg)	Specific Activity mUnits/g protein	Yield (%)	Overall Purification Factor*
105 000 x g Supernatant	184	14 620	12.6	100	1.0
DE 52 Cellulose Chromatography	175	3 360	52.1	95	4.1
Phosphocellulose P 11 Chromatography					
Unadsorbed (Tdr K I)	32.4	2 350	13.8	18	1.1
Adsorbed (Tdr K II)	152	410	371	83	29.4
CM Sephadex Chromatography of Tdr K I					
Unadsorbed (Tdr K IA)	20	1 700	16.5	15	1.3
Adsorbed (Tdr K IB)	2.3	7	329	1	26.1
Biogel A 1.5 Gel Filtration of Tdr K IA	13	490	26.5	7	2.1
Biogel A 1.5 Gel Filtration of Tdr K II					
(Tdr K IIA)	7.4	19	390	4	31
(Tdr K IIB)	59	96	615	32	48.8

* The overall purification factor compares the specific activity of the Tdr kinase fraction or fractions at each stage of the purification procedure with the specific activity of the mixture of Tdr kinases in the crude fraction. This therefore represents a minimum purification factor for each of the Tdr kinase forms.

TABLE 14
PARTIAL PURIFICATION OF HUMAN HEPATOMA THYMIDINE KINASES

Purification Stage	Total Activity (mUnits)	Total Protein (mg)	Specific Activity mUnits/g protein	Yield (%)	Overall Purification Factor*
105 000 x g Supernatant	184	14 620	12.6	100	1.0
DE 52 Cellulose Chromatography	175	3 360	52.1	95	4.1
Phosphocellulose P 11 Chromatography					
Unadsorbed (Tdr K I)	32.4	2 350	13.8	18	1.1
Absorbed (Tdr K II)	152	410	371	83	29.4
CM Sephadex Chromatography of Tdr K I					
Unadsorbed (Tdr K IA)	28	1 700	16.5	15	1.3
Absorbed (Tdr K IB)	2.3	7	329	1	26.1
Biogel A 1.5 Gel Filtration of Tdr K IA	13	490	26.5	7	2.1
Biogel A 1.5 Gel Filtration of Tdr K II (Tdr K IIA) (Tdr K IIB)	7.4 59	19 96	390 615	4 32	31 48.8

* The overall purification factor compares the specific activity of the Tdr kinase fraction or fractions at each stage of the purification procedure with the specific activity of the mixture of Tdr kinases in the crude fraction. This therefore represents a minimum purification factor for each of the Tdr kinase forms.

TABLE 15

THE RELATIVE PROPORTIONS OF THE THYMIDINE KINASE
FORMS IN HUMAN NORMAL LIVER AND HEPATOMA

Tdr Kinase Form	Normal Liver	Hepatoma
IA	17%	16%
IB	3%	2%
IIA	7%	9%
IIB	73%	73%

human normal liver and hepatoma. Tdr kinase IIB was the major form of the enzyme, accounting for 73% of human normal liver and hepatoma Tdr kinase activities. Tdr kinase IA accounted for about 16-17% of the activity in human normal and cancerous liver, and the remaining approximately 10% of the total activity was due to the Tdr kinase IB and IIA forms of the enzyme.

The activities of the contaminating enzymes thymidylate kinase, thymidylate phosphatase, and adenosine triphosphatase in the crude fraction and at the final purification stage for each of the Tdr kinase preparations, are summarised in Tables 16 and 17, for human normal liver and hepatoma respectively. Thymidylate kinase activity was absent from all of the Tdr kinase preparations. There was some contamination of Tdr K IA preparations by thymidylate phosphatase. ATPase activity, although substantially reduced, showed relatively high activity in most of the purified Tdr kinase preparations from human normal and cancerous liver, being particularly high in the Tdr kinase IA preparations.

Characterisation of Human Liver and Hepatoma Thymidine Kinases

Certain physical and kinetic properties of the Tdr kinase preparations from human normal liver and hepatoma were investigated to determine differences between the different enzyme forms in each tissue, and between the corresponding enzyme forms from normal and cancerous human liver.

Molecular Weights

The calibration curve for the estimation of the molecular weights of the different Tdr kinase forms is shown in Figure 19. The molecular weights for the Tdr kinase preparations from human adult liver and hepatoma tissues were: Tdr K IA - 130 000; Tdr K IB - 105 000; Tdr

TABLE 16

ACTIVITIES OF THYMIDYLATE KINASE, THYMIDYLATE PHOSPHATASE
AND ADENOSINE TRIPHOSPHATASE OBTAINED IN THE CRUDE FRACTION
AND AT THE FINAL PURIFICATION STAGE OF THE Tdr KINASE FORMS
FROM HUMAN NORMAL LIVER

Contaminating Enzyme	Activities of Normal Liver Tdr kinase Forms*				
	Crude Fraction	IA	IB	IIA	IIB
Thymidylate kinase	0	0	0	0	0
Thymidylate phosphatase	497%	73%	0	22%	0
Adenosine triphosphatase	26 638%	2 289%	0	0	249%

* Activities are expressed as a percentage of the thymidine kinase activity at the corresponding stage.

TABLE 17

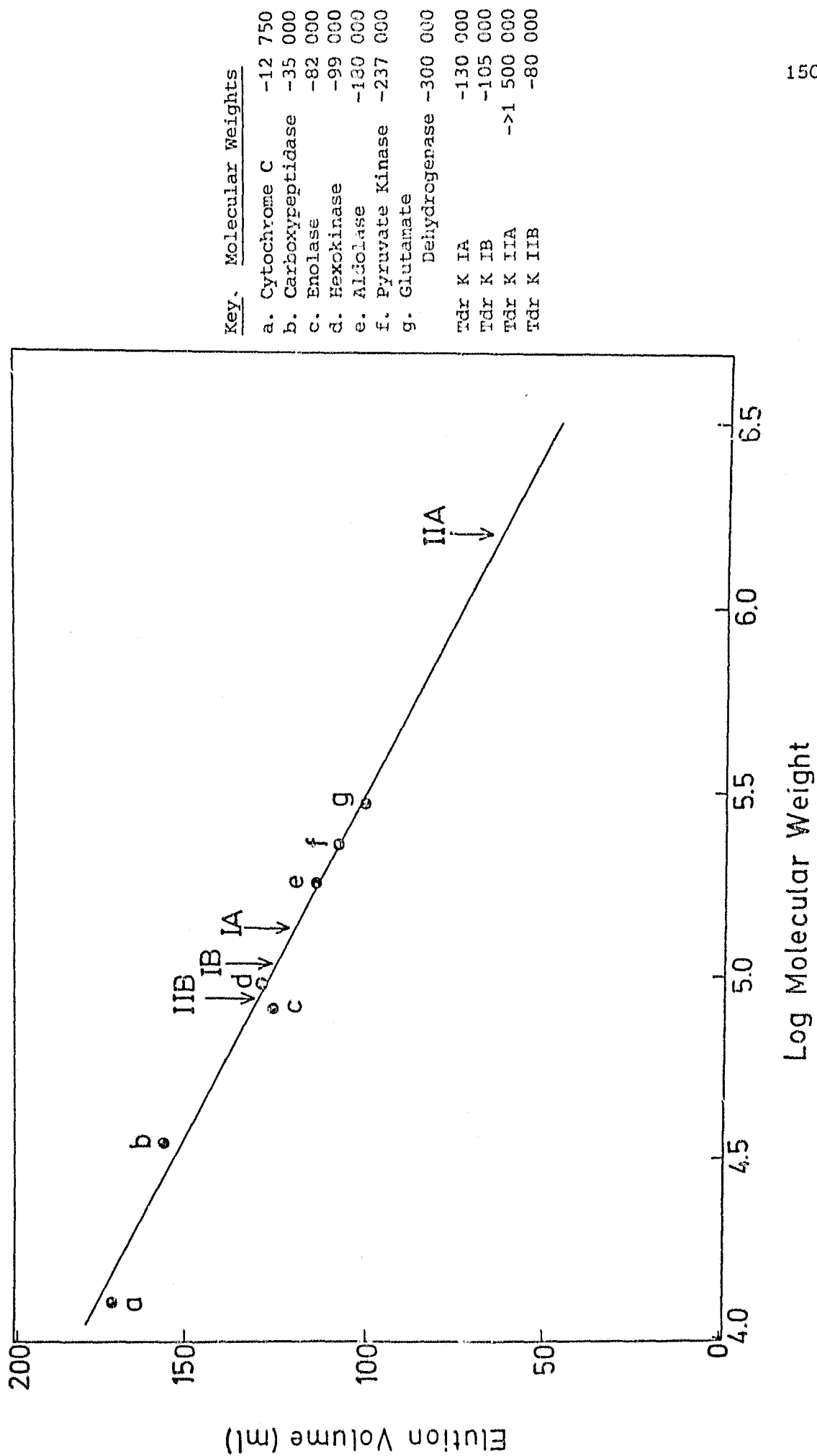
ACTIVITIES OF THYMIDYLATE KINASE, THYMIDYLATE PHOSPHATASE
AND ADENOSINE TRIPHOSPHATASE OBTAINED IN THE CRUDE
FRACTION AND AT THE FINAL PURIFICATION STAGES OF
THE Tdr KINASE FORMS FROM HUMAN HEPATOMA

Contaminating Enzyme	Activities of Hepatoma Tdr kinase Forms*				
	Crude Fraction	IA	IB	IIA	IIB
Thymidylate kinase	0	0	0	0	0
Thymidylate phosphatase	346%	469%	0	0	4%
Adenosine triphosphatase	36 263%	4 808%	798%	190%	67%

* Activities are expressed as a percentage of the thymidine kinase activity at the corresponding stage.

FIGURE 19

CALIBRATION CURVE FOR THE DETERMINATION OF THE MOLECULAR WEIGHTS OF THE TDR KINASE
ENZYME FORMS FROM HUMAN NORMAL AND CANCEROUS LIVER USING A BIOGEL A1.5 COLUMN



The column dimensions were 2 x 56cm. Proteins of known molecular weight (see Key above) and the Tdr kinase forms, were eluted using 20mM Tris-HCl buffer, pH 8.0, containing 10% glycerol, 10mM β -mercaptoethanol and 100mM KCl.

K IIA - greater than 1 500 000; Tdr K IIB - 80 000. In the case of Tdr K IIA, the enzyme preparation eluted in the void volume of the Biogel column.

Magnesium Requirements

To determine the ratio of magnesium to ATP required for optimum Tdr kinase activity, the activity of a partially-purified Tdr kinase preparation from human adult liver was estimated as a function of magnesium chloride concentration, in the presence of various constant ATP levels. All experiments were carried out using 50mM Tris-HCl buffer at pH 8.0. The results obtained suggested that the Mg:ATP ratio is dependent on the ATP concentration. At ATP levels of between 0.1 and 1.0mM an optimal Mg:ATP ratio of 1:1 was obtained; at ATP levels below 0.05mM the optimal Mg:ATP ratio increased to 5:1. Since the ATP concentrations used in the present study were generally equal to or greater than 0.1mM, a Mg:ATP ratio of 1:1 was used.

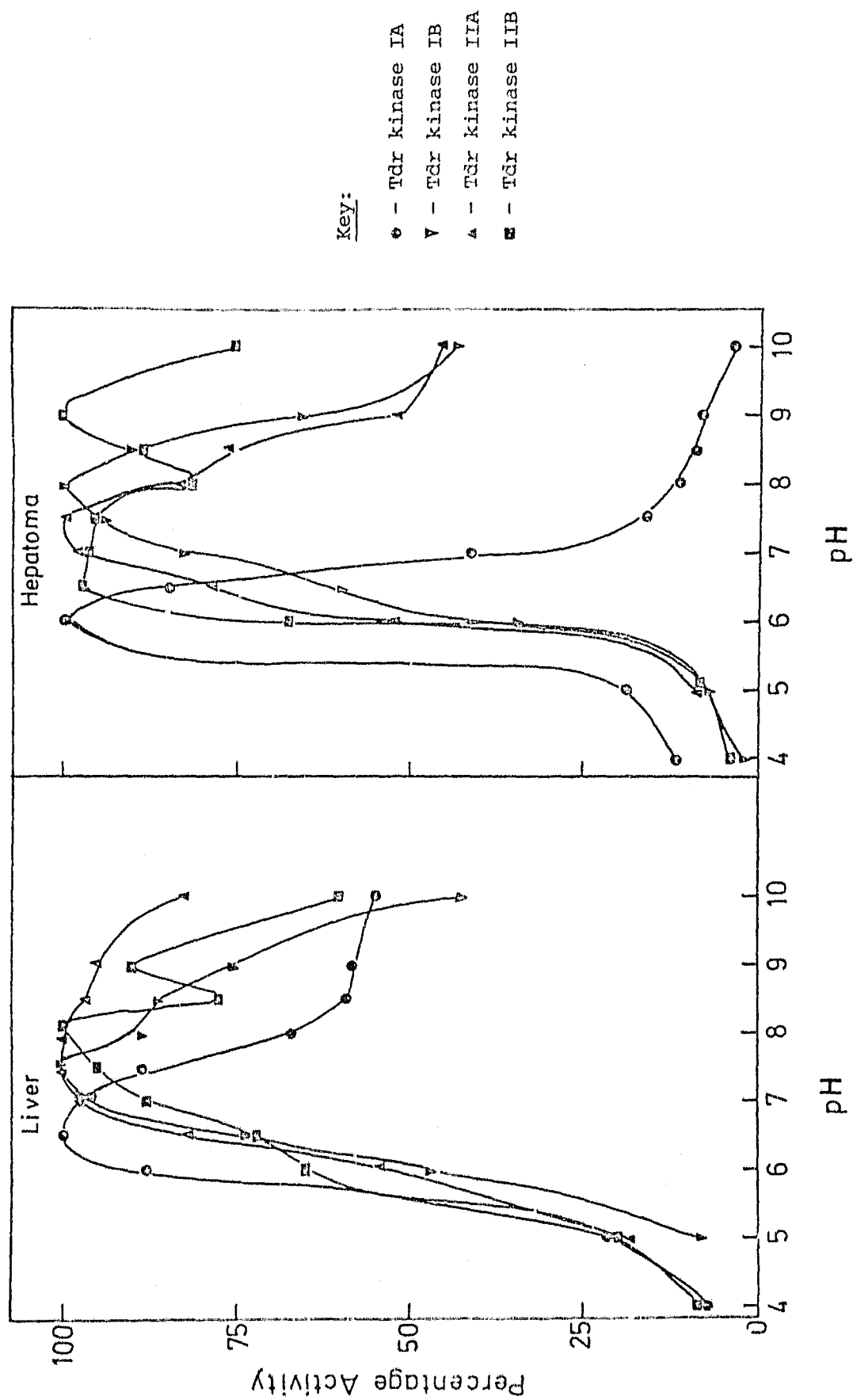
pH Optimum Studies

From plots of initial velocity versus pH (Figure 20) it can be seen that each of the liver or hepatoma Tdr kinase forms exhibited a characteristic pH optimum. The normal liver and hepatoma Tdr K IIB preparations gave biphasic pH optima, whereas those for the other forms of the enzyme were monophasic. The pH optima for the different Tdr kinase forms are summarised in Table 13, from which it can be seen that the values for the corresponding forms of Tdr kinase in normal liver and hepatoma were similar.

Thermostability Studies

Each of the Tdr kinase preparations was diluted two-fold with 20mM Tris-HCl buffer, pH 8.0, containing 20% glycerol and 10mM β -mercap-

FIGURE 20
pH OPTIMUM STUDIES ON HUMAN LIVER AND HEPATOMA Tdr KINASES



Assay mixtures contained: 50mM Tris-glycine-cacodylate buffer which had been adjusted to the required pH with HCl or NaOH; 250μM ATP; 250μM MgCl₂; 50μM 3H-Tdr (containing 200mCi/mmol); 37.5mM NaF; and suitably diluted enzyme preparation, in a final volume of 200μl. Results are expressed as a percentage of the highest activity obtained for each of the enzyme forms.

TABLE 18

pH OPTIMA FOR Tdr KINASES IN HUMAN NORMAL LIVER AND HEPATOMA

Tdr kinase form	Normal liver	Hepatoma
IA	6.5	6.0
IB	7.5	8.0
IIA	7.5 - 8.0	7.5
IIB	8.0 & 9.0	6.5 - 7.5 & 9.0

toethanol, or with the same buffer containing, in addition, 100 μ M thymidine. The enzyme preparations were incubated at 55°C for varying periods of time up to 20 minutes, after which aliquots were withdrawn and assayed for Tdr kinase activity. Figures 21 and 22 show, respectively, the results obtained for the normal liver and hepatoma Tdr kinase preparations. It can be seen that the Tdr kinase forms from human normal and cancerous liver were generally unstable to heating at 55°C. The most stable of the Tdr kinase forms was the normal liver IIA preparation, which retained about 40% of its original activity after incubation at 55°C for 10 minutes. Each of the other forms lost at least 75% of its initial activity after heating at 55°C for 10 minutes. The presence of Tdr (100 μ M) during the 55°C incubation partially protected the normal liver and hepatoma Tdr kinase forms from inactivation.

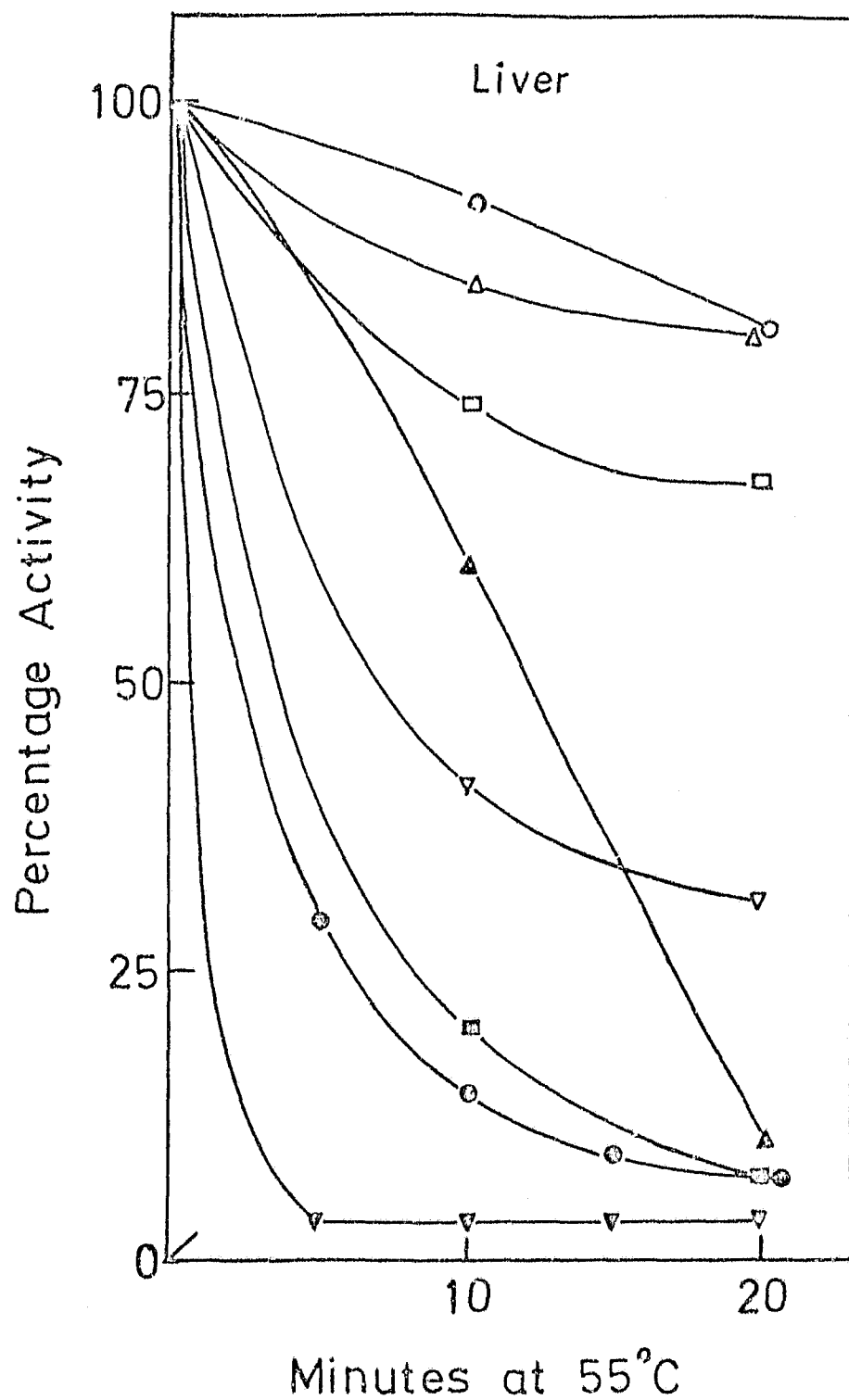
Ammonium Sulphate Inhibition

Figure 23 illustrates the effect of ammonium sulphate at concentrations in the range of 10-150mM on the activity of Tdr kinases from human normal liver and hepatoma. In general all Tdr kinase forms were inhibited to a similar extent by the presence of ammonium sulphate.

Michaelis Constants for Thymidine

The apparent Michaelis constants for thymidine for liver and hepatoma thymidine kinase preparations were obtained from Lineweaver-Burk plots of reciprocal initial velocity versus reciprocal Tdr levels, at constant (100 μ M) Mg-ATP concentrations (Figures 24 to 27). Table 19 summarises the apparent K_m values obtained using the computer analysis described under the Methods section. It can be seen that the constants were similar for the corresponding Tdr kinase forms from normal liver and hepatoma. The apparent K_m value for Tdr K 1A was found to be

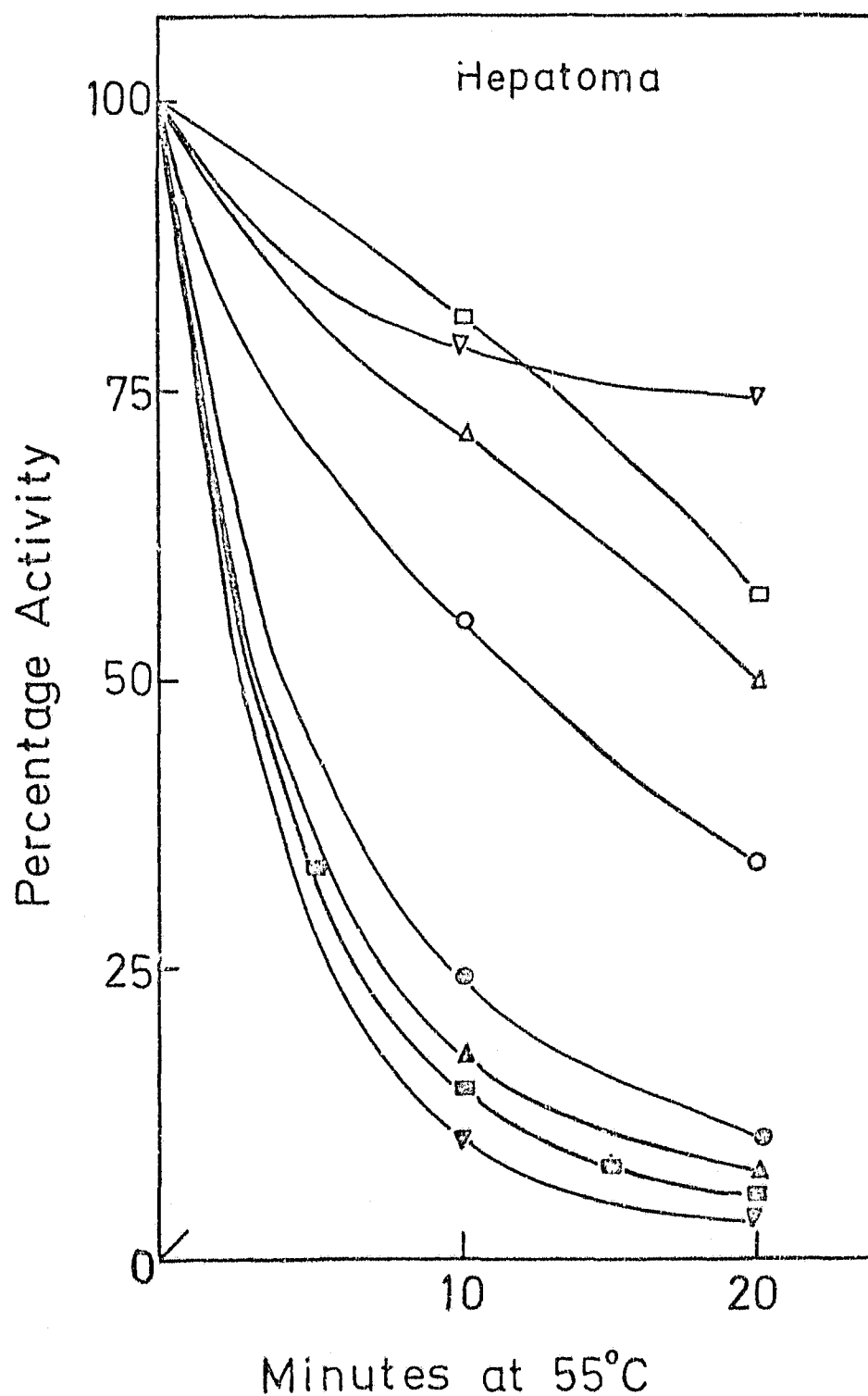
THE EFFECT OF HEATING NORMAL LIVER TDR KINASE ENZYME FORMS
AT 55°C FOR VARIOUS TIME INTERVALS IN THE PRESENCE AND
ABSENCE OF THYMIDINE



Key: ○ - Tdr K IA; ▽ - Tdr K IB; Δ - Tdr K IIA; □ - Tdr K IIB.

Enzyme preparations were incubated without Tdr (●) or in the presence of 100μM Tdr (○). Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100μM ATP; 100μM MgCl₂; 37.5mM NaF; 50μM ³H-Tdr (containing 200mCi/mmol); and 100μl of the incubated enzyme preparation, in a final volume of 200μl. In the case of Tdr kinase forms pre-incubated with 100μM Tdr only ³H-Tdr (final concentration of 200mCi/mmol) was added to the assay mixture. Results are expressed as a percentage of the activity of each enzyme preparation prior to incubation at 55°C.

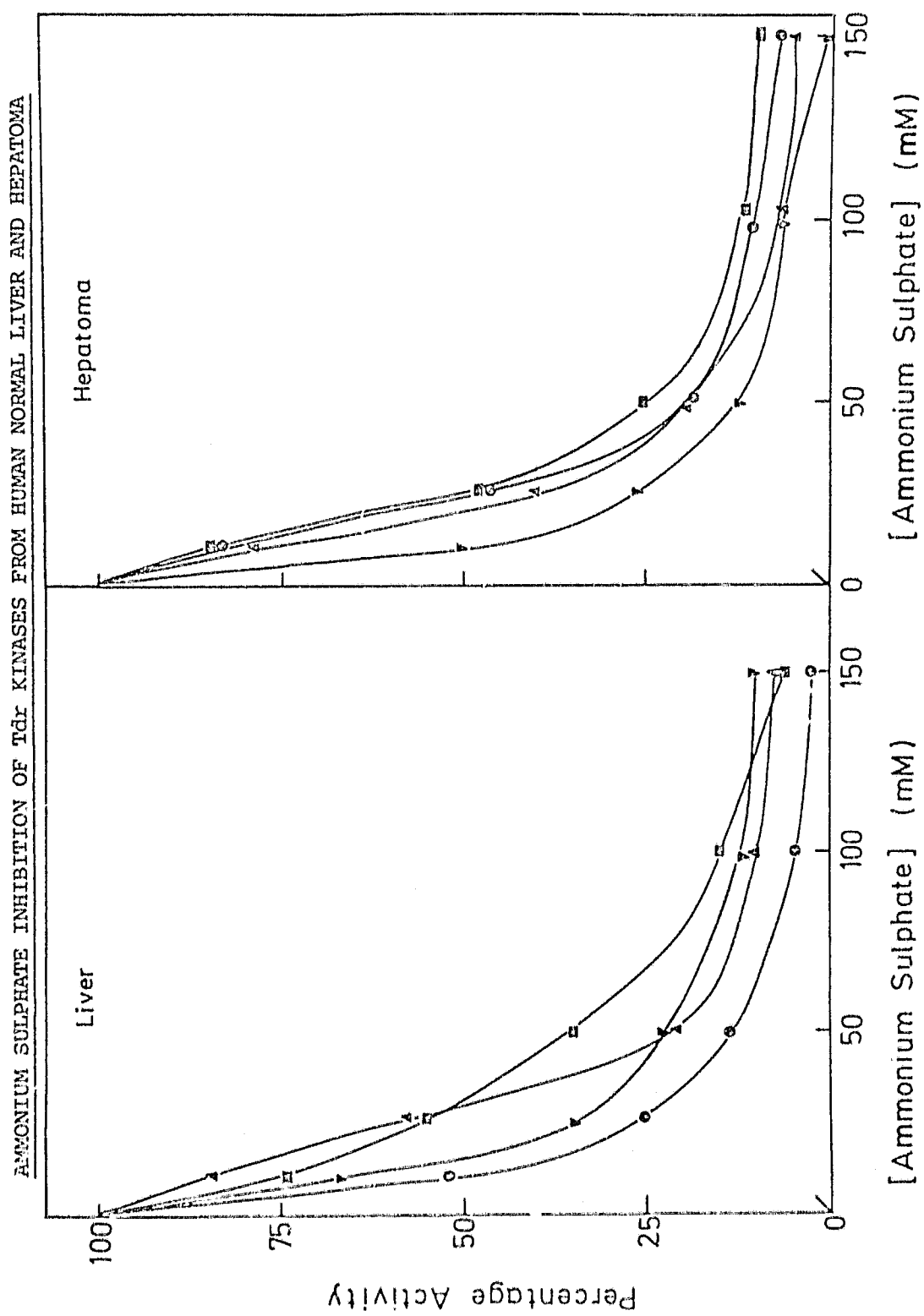
THE EFFECT OF HEATING HEPATOMA Tdr KINASE ENZYME FORMS AT 55°C FOR VARIOUS TIME INTERVALS IN THE PRESENCE AND ABSENCE OF THYMIDINE



Key: ○ - Tdr K IA; □ - Tdr K IB; ▽ - Tdr K IIA; △ - Tdr K IIB.

Enzyme preparations were incubated without Tdr (○) or in the presence of 100µM Tdr (●). Details of the assay mixture used are described in Figure 21. Results are expressed as a percentage of the activity of each enzyme preparation prior to incubation at 55°C.

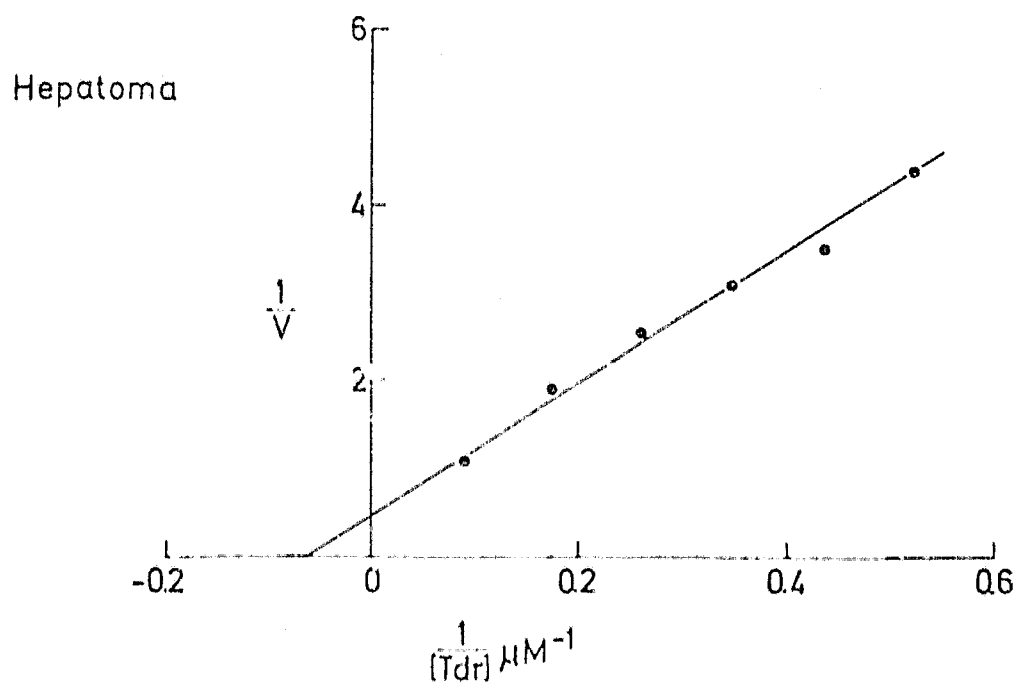
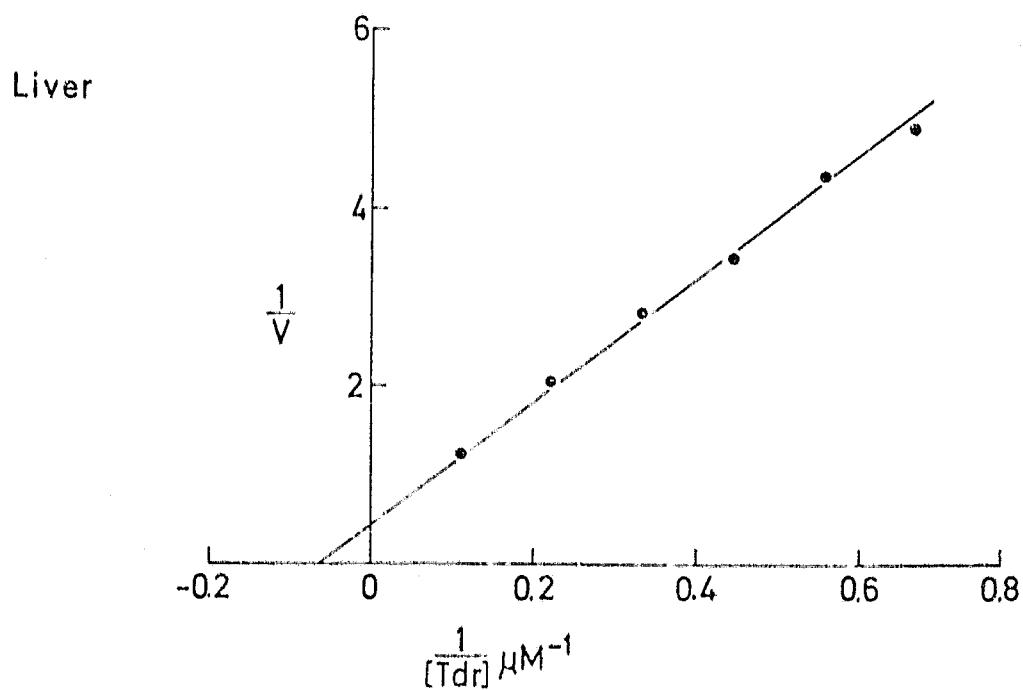
FIGURE 23



Key: ● - Tdr K IA; ▼ - Tdr K IB; ▲ - Tdr K IIA; ■ - Tdr K IIB.
 Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100mM ATP; 100mM MgCl₂; 37.5mM NaF; 50μM ³H-Tdr (containing 2000Ci/mole); ammonium sulphate as indicated; and enzyme preparation, in a final volume of 200μl. Results are expressed as the percentage activity of each preparation relative to the activity measured in the absence of ammonium sulphate.

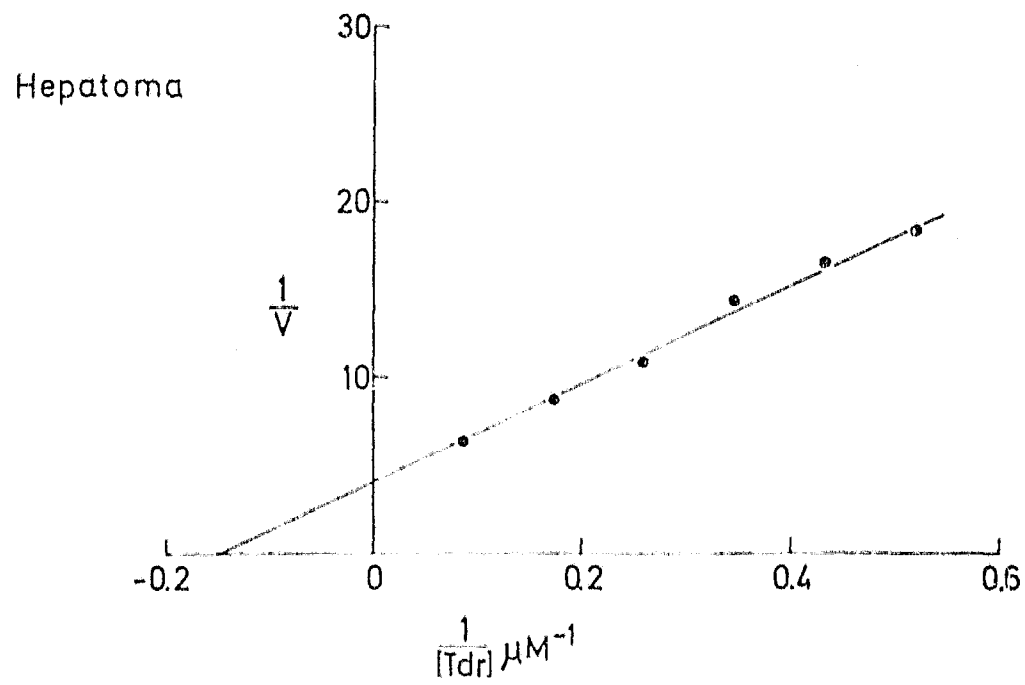
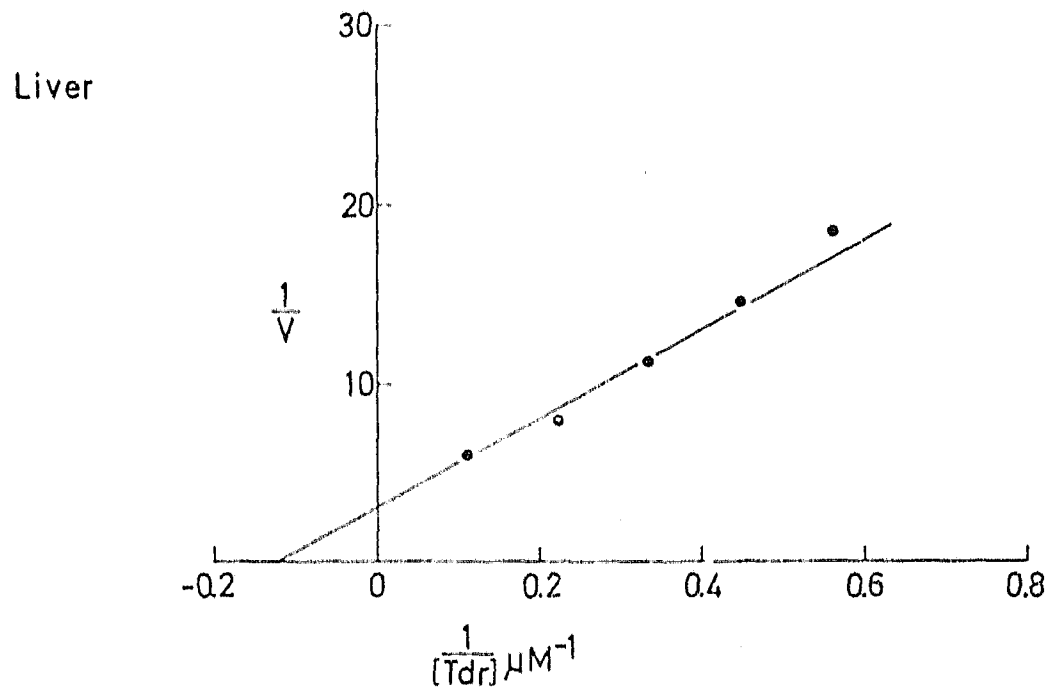
FIGURE 24

LINEWEAVER-BURK PLOTS OF RECIPROCAL VELOCITY VERSUS RECIPROCAL
Tdr CONCENTRATION FOR Tdr KINASE TA FROM HUMAN NORMAL LIVER AND
HEPATOMA



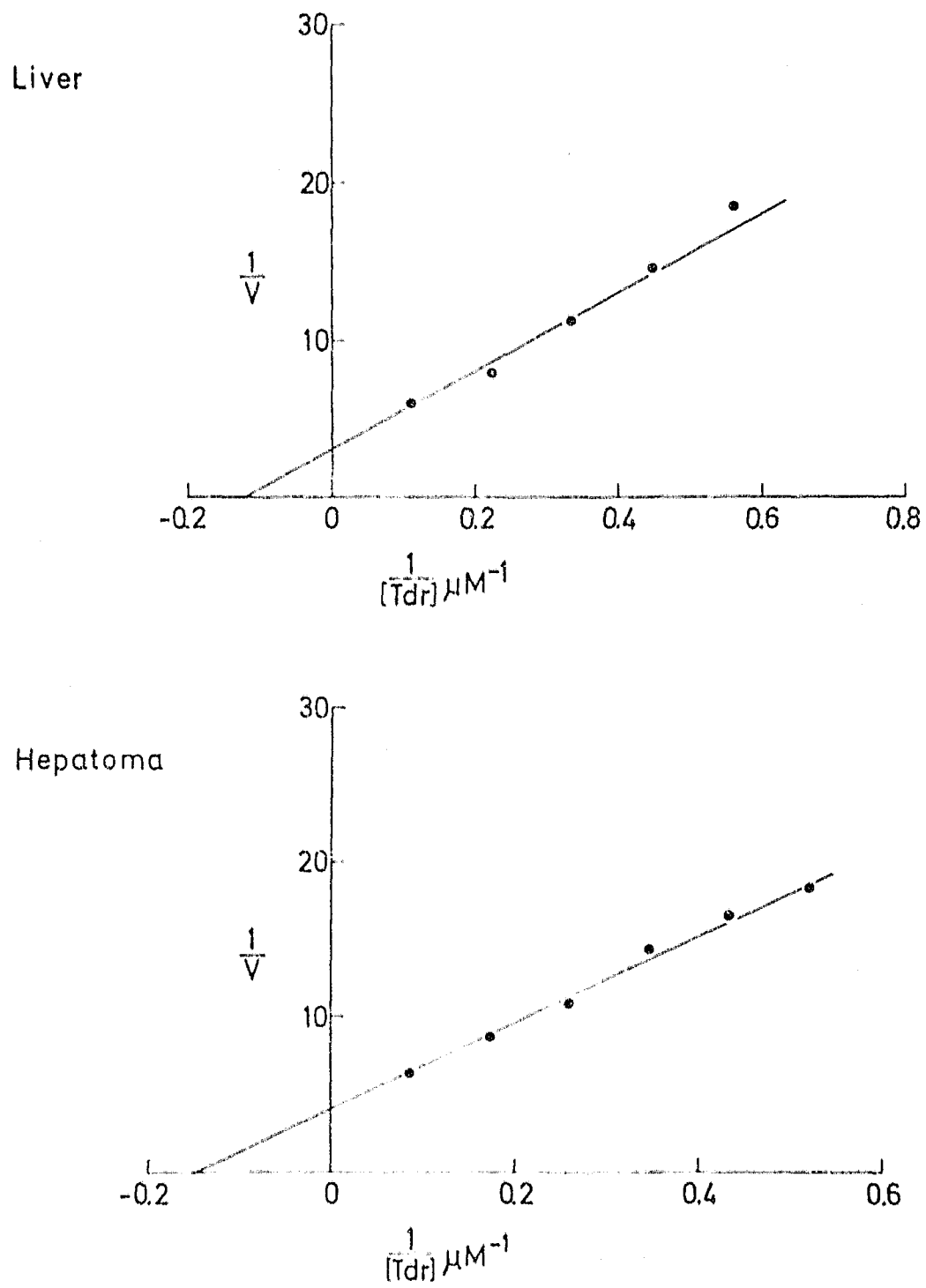
Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100μM ATP; 100μM MgCl₂; 37.5mM NaF; varying concentrations of ³H-Tdr; and suitable dilutions of enzyme preparation, in a final volume of 200μl.

LINEWEAVER-BURK PLOTS OF RECIPROCAL VELOCITY VERSUS RECIPROCAL Tdr
CONCENTRATION FOR Tdr KINASE 1B FROM HUMAN NORMAL LIVER AND HEPATOMA



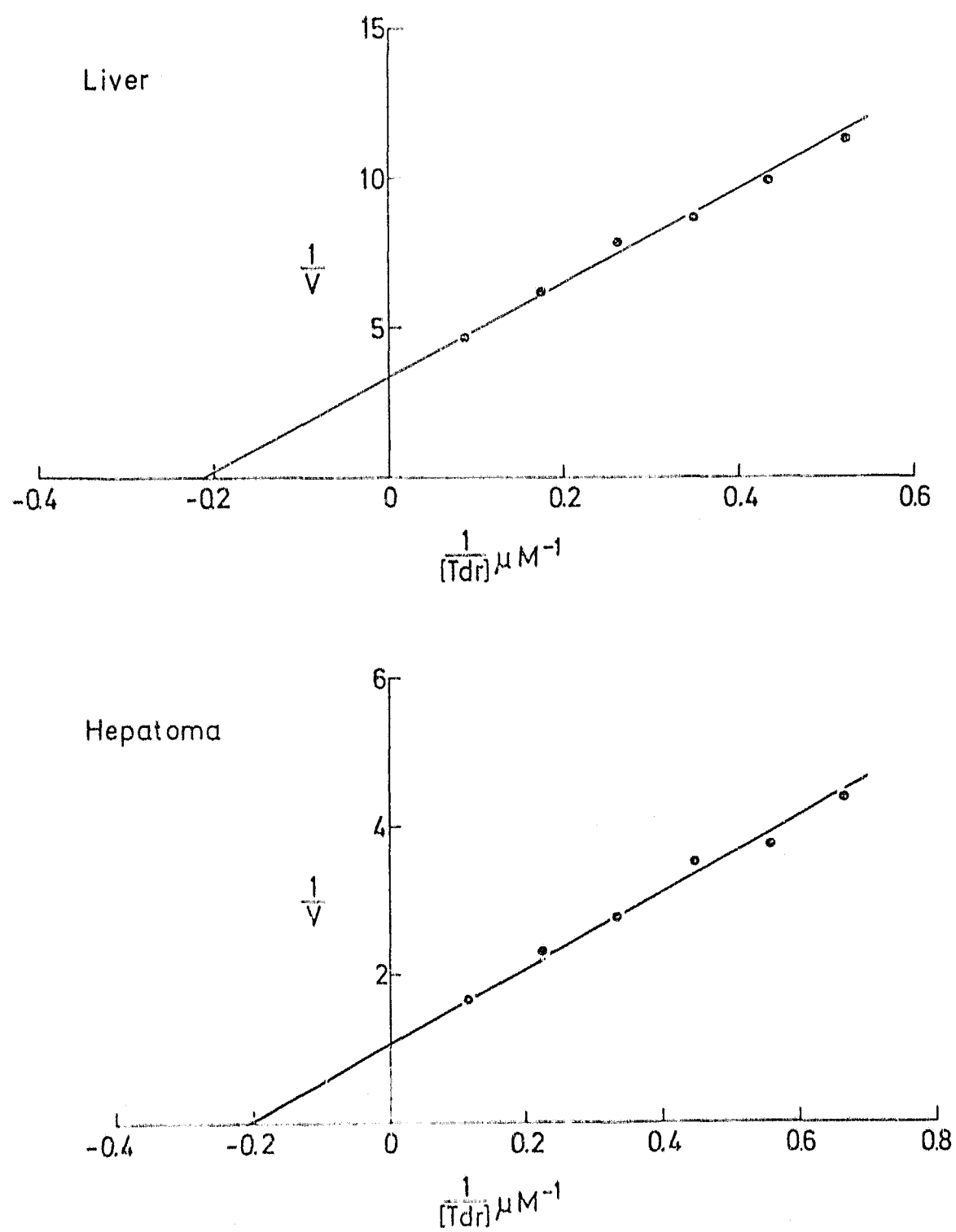
Assay conditions were as described in Figure 24.

LINEWEAVER-BURK PLOTS OF RECIPROCAL VELOCITY VERSUS RECIPROCAL Tdr CONCENTRATION FOR Tdr KINASE 1B FROM HUMAN NORMAL LIVER AND HEPATOMA



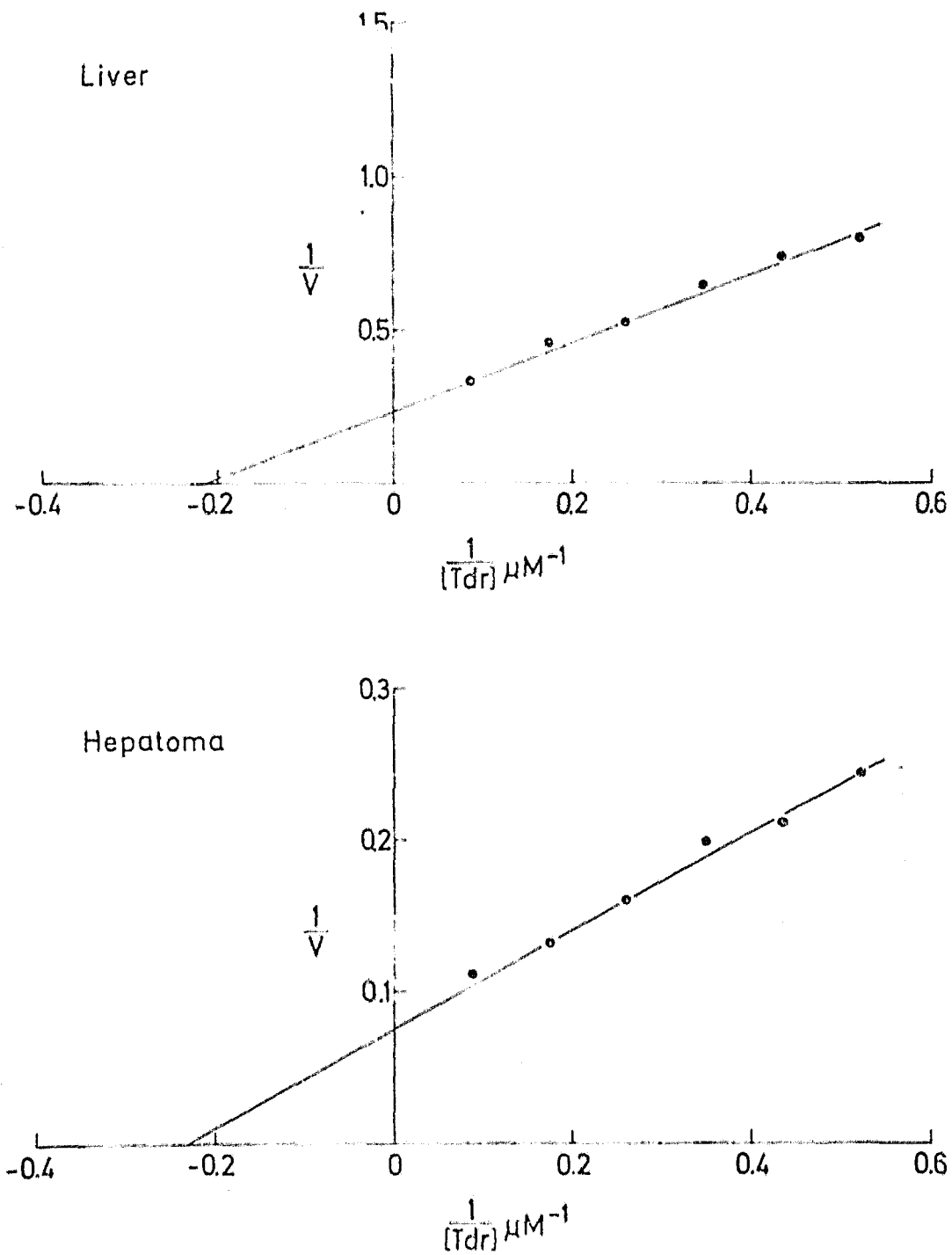
Assay conditions were as described in Figure 24.

LINEWEAVER-BURK PLOTS OF RECIPROCAL VELOCITY VERSUS RECIPROCAL Tdr CONCENTRATION FOR Tdr KINASE IIA FROM HUMAN NORMAL LIVER AND HEPATOMA



Assay conditions were as described in Figure 24.

LINEWEAVER-BURK PLOTS OF RECIPROCAL VELOCITY VERSUS RECIPROCAL Tdr
CONCENTRATION FOR Tdr KINASE IIB FROM HUMAN NORMAL LIVER AND HEPATOMA



Assay conditions were as described in Figure 24.

TABLE 19

SUMMARY OF THE APPARENT K_m VALUES FOR THYMIDINE FOR Tdr
KINASES FROM HUMAN NORMAL AND CANCEROUS LIVER

Tdr Kinase Form	[ATP] _{in} M	Apparent Michaelis Constants (μ M)	
		Normal Liver	Hepatoma
IA	0.1	15.5 $\pm$ 1.9	17.3 $\pm$ 2.3
IB	0.1	8.4 $\pm$ 3.1	6.9 $\pm$ 0.9
IIA	0.1	4.8 $\pm$ 0.5	4.8 $\pm$ 0.4
IIB	0.1	4.9 $\pm$ 0.5	4.5 $\pm$ 0.4
IA	0.5	11.7 $\pm$ 1.2	

K_m values and standard errors for each determination
 are listed.

approximately twice that of Tdr K IB; and four times those of Tdr K's IIA and IIB, which were similar. Since the Tdr kinase IA preparations showed high contamination by ATPase, the K_m for Tdr was also evaluated in the presence of a higher (500 μ M) concentration of Mg-ATP. It can be seen from Table 19 that this produced only a slight decrease in the apparent K_m for Tdr of the Tdr kinase IA preparation.

Michaelis constants for Mg-ATP

Since some of the fractions were contaminated with ATPase, reliable Michaelis constants for Mg-ATP could not be obtained.

Nucleoside and Deoxynucleoside Triphosphates as Phosphate Donors

Table 20 shows the percentage activity obtained with human normal liver and hepatoma Tdr kinases on replacing ATP with equivalent concentrations of dATP, CTP, dCTP, GTP, dGTP, dTTP, or UTP. It can be seen that the corresponding normal liver and hepatoma Tdr kinase preparations used a particular nucleoside or deoxynucleoside triphosphate to a similar extent. dTTP did not serve as a phosphate donor. On the other hand CTP was found to be equally effective as, if not more effective than, ATP as a phosphate donor. dATP, dCTP, GTP, dGTP, and UTP were utilised to a lesser extent.

Nucleotide and Deoxynucleotide Inhibitors

The results of assaying human normal liver and hepatoma Tdr kinase preparations in the presence of 100 μ M ATP together with equimolar amounts of CTP, dCTP, dTTP, dTDP, dTTP, dATP, dUMP, dGTP, or UTP are summarised in Table 21. The inhibitory effect of a particular nucleotide or deoxynucleotide was similar when comparing the corresponding forms of thymidine kinase in normal liver and hepatoma, and when comparing the enzyme forms within a given tissue. dTTP and dTDP were potent inhibitors, giving 82-83 inhibition. dATP gave somewhat lower inhibition

TABLE 20
UTILISATION OF NUCLEOSIDE AND DEOXYNUCLEOSIDE TRIPHOSPHATES AS PHOSPHATE
DONORS BY Tdr KINASE PREPARATIONS FROM HUMAN NORMAL AND CANCEROUS LIVER

Phosphate Donor	Percentage Activity							
	Tdr kinase forms in normal liver				Tdr kinase forms in hepatoma			
	IA	IB	IIA	IIB	IA	IB	IIA	IIB
ATP	100	100	100	100	100	100	100	100
dATP	22	18	35	25	22	17	17	31
CTP	107	139	113	89	87	111	108	105
dCTP	15	10	18	16	11	7	13	28
GTP	35	64	59	45	42	70	56	41
dGTP	47	59	61	39	26	58	52	50
dTTP	2	0	0	0	0	0	3	3
UTP	29	58	53	33	23	52	48	40

Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100mM MgCl₂; 50mM ³H-Tdr (containing 200mCi/mmol); 37.5mM NaF; 100mM nucleoside or deoxynucleoside triphosphate as indicated; and enzyme preparation, in a final volume of 200μl.

Results are expressed as percentage enzyme activity relative to the activity obtained using ATP as the phosphate donor.

TABLE 21

INHIBITION OF TdR KINASES FROM HUMAN NORMAL AND CANCEROUS
LIVER BY NUCLEOSIDE AND DEOXYNUCLEOSIDE PHOSPHATES

Inhibitor	Percentage Inhibition								
	TdR kinase forms in normal liver				TdR kinase forms in hepatoma				
	IA	IB	IIA	IIB	IA	IB	IIA	IIB	
CTP	0	0	11	0	0	0	0	0	0
dCTP		88	89	75	76	91	88	78	
ATP	43	49	43	29	63	61	37	24	
dATP	96	88	82	90	93	92	89	95	
UTP	97	94	84	93	98	89	97	94	
dUTP	64	79	77	57	69	84	76	63	
GMP	0	0	5	6	16	7	0	2	
dGTP	0	0	13	10	0	0	0	14	
UTP	7	6	17	16	21	7	0	12	

Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100 μ M ATP; 100 μ M MgCl₂; 37.5mM NaF; 50 μ M ³H-TdR (containing 200mCi/mmol); 100 μ M nucleotide or deoxynucleotide as indicated; and enzyme preparation, in a final volume of 200 μ l.

Results are expressed as percentage inhibition of enzyme activity relative to activity measured in the presence of ATP alone.

(57-84%) and dTMP still less (29-63%). The least inhibitory analogues were CTP, UTP, dGTP, and dUMP, with 0-21% inhibition.

Thymidine Analogue Studies

The ability of various thymidine analogues to serve as substrates for Tdr kinase was investigated using a crude supernatant preparation from human normal liver (ie. predominantly Tdr kinase IIB). Activity was determined as described on page 125. The results obtained are summarised in Table 22. Thymine and uracil (which lack deoxyribose moieties) and uridine were not effective as substrates. On the other hand deoxyuridine was a more efficient substrate than thymidine. 5-Fluoro-uridine and cytidine were relatively poor substrates, whereas 5-fluoro-deoxyuridine and deoxycytidine were comparable with thymidine. In contrast to 5-fluoro-deoxyuridine, 5-bromo-deoxyuridine and 5-iodo-deoxyuridine were utilised to a lesser extent than thymidine.

The effects of these thymidine analogues on the activities of the Tdr kinase forms from human liver and hepatoma, when assayed in the presence of thymidine, are summarised in Table 23. In each assay the concentration of the analogue was five-fold higher than that of Tdr. Thymidine produced little or no inhibitory effect on the Tdr K IB, IIA, or IIB preparations, but led to 47-69% inhibition of the Tdr K IA preparations. Uracil produced a smaller inhibitory effect on the Tdr K IA preparations, with little or no effect on the other Tdr kinase forms. The presence of uridine and deoxyuridine had little or no inhibitory effect on the human liver or hepatoma Tdr kinase activities. 5-Fluoro-, 5-bromo-, and 5-iodo-deoxyuridine produced 35-71% inhibition of each of the Tdr kinase forms, with the exception of the Tdr K IA forms of the enzyme. In the latter case, enzyme activity of the form from liver was inhibited by only 10%, while activity of the hepatoma form was increased

TABLE 22
THE ABILITY OF THYMIDINE ANALOGUES TO SERVE AS
SUBSTRATES IN THE Tlr KINASE CATALYSED REACTION

Substrate	Percentage Activity
Thymidine	100
Thymine	5
Uracil	5
Uridine	0
Deoxyuridine	200
5-Fluoro-uridine	15
5-Fluoro-deoxyuridine	111
5-Iodo-deoxyuridine	46
5-Bromo-deoxyuridine	47
Cytidine	38
Deoxycytidine	94

Each assay mixture contained: 50 μ M Tris-HCl buffer, pH 8.0; 100 μ M ^{32} P-ATP (containing 20mCi/ μ mol); 400 μ M MgCl₂; 20 μ M test substrate as indicated; and enzyme preparation, in a final volume of 200 μ l.

Results are expressed as percentage activity relative to the activity measured with Tlr as the substrate.

TABLE 23
EFFECTS OF THYMIDINE ANALOGUES ON
HUMAN LIVER AND HEPATOMA TDR KINASES

Analogue	Percentage Decrease in Enzyme Activity in the Presence of Analogue							
	Tdr kinase forms in normal liver				Tdr kinase forms in hepatoma			
	IA	IB	IIA	IIB	IA	IB	IIA	IIB
Thymine	47	21	4	6	69	0	10	6
Uracil	19	10	5	0	33	0	1	0
Uridine	0	13	0	0	11	5	12	0
Deoxyuridine	0	13	5	7	0	17	17	11
5-Fluoro-deoxyuridine	10	40	42	38	† 51	34	31	36
5-Bromo-deoxyuridine	62	70	71	67	31	65	64	69
5-Iodo-deoxyuridine	38	48	35	35	47	58	31	37
Cytidine	0	2	5	0	18	0	10	7
Deoxycytidine	69	79	76	60	62	75	86	69

Each assay mixture contained: 50mM imidazole-HCl buffer, pH 6.5; 100μM ATP; 100μM MgCl₂; 37.5mM NaF; 50μM 3H-Tdr (containing 200mCi/mmol); enzyme preparation; and 250μM Tdr analogue as indicated, in a final volume of 200μl.

Results are expressed as the percentage decrease (or increase, †) in Tdr kinase activity in the presence of each analogue, relative to the activity measured in the absence of the analogue.

in the presence of the analogue. Cytidine had little or no effect on the activities of any of the Tdr kinase preparations, whereas the presence of deoxycytidine resulted in 60-86% inhibition of the liver and hepatoma Tdr kinase forms.

DISCUSSION

PURIFICATION AND CHARACTERISATION OF THYMIDINE KINASE FORMS FROM HUMAN LIVER AND HEPATOMA TISSUES

Due to the advanced and disseminated stage of liver carcinoma development in the majority of Black patients presenting with this disease surgery is seldom undertaken, and it was not possible to obtain any surgically removed hepatoma material during the course of these purification studies. The normal and cancerous liver tissues used were, however, obtained within as short a period of time as possible after death.

Pilot Purification Studies

The purification procedure described by Taylor *et al.* (1972) for human adult and foetal liver Tdr kinases was initially attempted using human normal liver and hepatoma tissues. Pilot studies showed that not all of the Tdr kinase activity in these tissues could be precipitated at ammonium sulphate saturation of between 0 and 40% as described by these authors. It was found that ammonium sulphate saturations of between 30 and 60% were necessary to precipitate Tdr kinase activities from human normal and cancerous liver tissues, and that this fractionation procedure resulted in a 50% loss of Tdr kinase activity and did not significantly reduce contaminating ATPase or dTMPase activities. This fractionation procedure was therefore not included in the purification procedures used in the present study.

Using 38 000 x g supernatants from human normal liver and hepatoma, a single peak of Tdr kinase activity was obtained on DE 52 cellulose chromatography. The finding of a single peak of Tdr kinase activity in human hepatoma differs from the finding of two peaks of enzyme activity in Morris rat hepatoma 7793 (Fujii *et al.*, 1972) and in human adreno-

cortical carcinoma (Nawata *et al.*, 1976).

On assaying the human hepatoma preparation for dTMP kinase, dTMPase, and ATPase contamination [in the latter case using the colorimetric method of Lowry (1957)] only dTMPase contamination was detectable. This contamination could be substantially reduced by passing the enzyme preparation through a BioGel A1.5 gel-filtration column, since dTMPase activity eluted ahead of the fractions containing Tdr kinase activity. The finding that considerable Tdr kinase activity was lost during the gel-filtration purification step is in agreement with the findings for Tdr kinase preparations from rat Walker carcinosarcomas (Bresnick and Thompson, 1965).

In the course of thymidine analogue studies using γ - ^{32}P -labelled ATP, as described on page 125, it was observed that high ^{32}P -inorganic phosphate was present in blanks from which the phosphate acceptors were omitted. This suggested the presence of ATP-hydrolysing activity in the enzyme preparation, although negative results for the presence of ATPase had been obtained using the colorimetric assay described by Lowry (1957). On assaying for ATPase activity with γ - ^{32}P -ATP as the substrate it was found that, although 90% of the contaminating activity present in a 30 000 x g supernatant had been removed during DEAE-cellulose chromatography, the specific activity of the residual ATPase was 250-fold greater than that of Tdr kinase in the preparation. It is therefore apparent that the use of the colorimetric assay of Lowry (1957) for the determination of ATPase contamination in Tdr kinase preparations is not sufficiently sensitive. This finding suggests that the Tdr kinase preparations of other workers who have estimated ATPase contamination by the method of Lowry (1957) may also have been contaminated by this enzyme.

Attempts to Reduce ATPase Contamination

In an attempt to reduce ATPase contamination, further purification of the human liver Tdr kinase preparation was carried out. It was found that centrifuging at 105 000 x g greatly reduced ATPase contamination of the Tdr kinase preparations. This probably represents the removal of ATPase activity associated with a microsomal fraction.

The ATPase contamination was further reduced by ion-exchange chromatography on Phosphocellulose P11 and CM-Sephadex. It was found that multiple forms of Tdr kinase could be separated using these procedures.

In addition to the above modifications, changes were made to the assay conditions for Tdr kinase to further reduce contaminating ATPase activity. These included the use of a lower pH buffer (6.5), and the addition of 37.5mM sodium fluoride to all assay mixtures. These changes did not affect the Tdr kinase activity.

The presence of 37.5mM sodium fluoride did not completely inhibit ATPase activity, a finding which differs from that reported for rat regenerating liver Tdr kinase preparations, where total inhibition of contaminating ATPase activity by 20mM sodium fluoride was obtained (Kizer and Holman, 1974). Levels of sodium fluoride higher than 37.5mM produced slight inhibition of human liver Tdr kinase activity, and it was therefore decided not to exceed this concentration. Although ouabain (50µM) has been reported to inhibit the sodium-potassium-activated form of ATPase from rabbit brain (Bonting *et al.*, 1964), this inhibitor had no effect on contaminating ATPase in the present study. This suggests that contamination may have been due to the calcium-magnesium-dependent form of the enzyme.

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Although the modifications to the purification and assay procedures for Tdr kinases from human normal and cancerous liver tissues did not eliminate ATPase activity, they considerably reduced contamination due to this enzyme, and simultaneously led to the separation of multiple forms of Tdr kinase activity from both tissues.

Purification of Tdr Kinases from Human Normal Liver and Hepatoma

The finding of at least four forms of Tdr kinase in human adult liver represents the first report of the existence of multiple Tdr kinase forms in this tissue. Taylor *et al.* (1972), who used only ammonium sulphate fractionation to partially purify their enzyme, have reported a single form of Tdr kinase in human adult liver. This probably corresponds to the single peak of enzyme activity eluted from a DEAE-cellulose column in the present study. The elution of only a single peak of Tdr kinase activity from DEAE-cellulose for human hepatoma preparations differs from the finding of two peaks of enzyme activity in human foetal liver and human cell-lines (Taylor *et al.*, 1972) and in rat hepatomas (Fujii *et al.*, 1972). No tumour-specific form of Tdr kinase was isolated from human hepatoma tissue, as has been found for human bladder adenocarcinoma, rhabdomyosarcoma, uterine fibroma, and HeLa and KB cell-line Tdr kinases (Stafford and Jones, 1972).

The relative proportions of the four Tdr kinase forms purified from human normal liver and hepatoma were similar in both tissues. In both normal and cancerous human liver Tdr kinase IIB, which accounted for about 75% of the total Tdr kinase activity, was the major enzyme form.

The similar Tdr kinase forms in human normal liver and hepatoma tissues suggest that the number of enzyme forms isolated from each tissue is not a result of proteolytic alterations. At this stage it is not clear whether the multiple forms of Tdr kinase activity in human normal

and cancerous liver tissues represent separate gene products (ie. isozymes) or are merely a result of post-translational modifications.

The pattern of contamination due to both ATPase and dTMPase in human liver Tdr kinases differed from that in human hepatoma Tdr kinases. The activity of ATPase assayed in the hepatoma 105 000 x g supernatant was approximately 40% higher than in the corresponding supernatant from human normal liver. This may explain why ATPase contamination was present in all of the hepatoma Tdr kinase preparations, but only in the IA and IIB fractions from normal liver. In addition the liver and hepatoma Tdr K IA preparations contained contaminating dTMPase activity. No dTMP kinase activity was present in any of the Tdr kinase preparations from normal or cancerous human liver. This is probably due to the fact that human liver dTMP kinase is labile to storage at -20°C (page 90).

Characterisation of the Tdr Kinase Forms from Human Normal

Liver and Hepatoma

Molecular Weights

The molecular weights of 130 000 and 105 000 for, respectively, the Tdr kinase IA and Tdr kinase IB preparations from both human normal and cancerous liver, are similar to those of 120 000 to 130 000 reported for the enzyme from rat regenerating liver (Walboomers and Wanka, 1974) and for the peak II (tumour specific) form of the enzyme from rat Yoshida sarcoma tissue (Fujii *et al.*, 1972 ; Hashimoto *et al.*, 1972). The lower molecular weight of 80 000 for the human liver and hepatoma Tdr kinase IIB preparations resembles those of 69 000 for rat regenerating liver Tdr kinase (Kizer and Holman, 1974), 70 000 for the peak I form of the enzyme from rat Yoshida sarcoma tissue (Fujii *et al.*, 1972 ; Hashimoto *et al.*, 1972) and 70 000 and 90 000 respectively for the mitochondrial and cytoplasmic forms of the enzyme from human leukemic blast cells

(Lee and Cheng, 1976b). The human liver and hepatoma Tdr kinase IIA preparations eluted in the void volume of the BioGel A1.5 column used, suggesting that these preparations have a molecular weight in excess of 1.5×10^6 . This high molecular weight may represent protein aggregation or may be due to association of this form of Tdr kinase with a non-protein high molecular weight compound. The latter possibility appears more likely, since Bresnick and Thompson (1965) have demonstrated the presence of a ribonucleic acid modifier associated with Tdr kinases from rat Walker carcinosarcomas and MDAB hepatomas.

Magnesium Requirements

Studies of the optimum ratios of magnesium to ATP were performed using a human liver preparation containing all four Tdr kinase forms. The finding that, at ATP concentrations of 100 μ M or higher, the optimum enzyme activity was obtained at Mg:ATP ratios of 1:1 is in agreement with the findings for human leukaemic blast cells (Lee and Cheng, 1976c) and for Tdr kinases induced in HeLa TK⁻ cells by Herpes simplex viruses Types I and II (Cheng, 1976).

At lower concentrations of ATP (20-50 μ M) it was found that the optimum Mg:ATP ratio for the human liver Tdr kinase preparation increased to 5:1. Morrison and Heyde (1972) have calculated that for equimolar concentrations of Mg and ATP (1mM) at pH 8.0, some 90% of the total ATP would be in the form of Mg-ATP. It can, however, be shown that at lower concentrations (eg. 20 μ M) of Mg and ATP only about 40% of the total ATP may be in the form of Mg-ATP. The total concentration of the Mg-ATP complex may thus be lower than the Michaelis constant for this substrate complex, or alternatively the higher levels of free ATP⁴⁻ may be inhibitory. Calculations show that increasing the Mg:ATP ratio to 5:1 at the lower ATP concentration may lead to about 80% of the total ATP

being complexed as Mg-ATP, thus avoiding the possibility of low substrate levels or inhibition due to large quantities of free ATP. This may explain the increased Mg:ATP ratio at low ATP levels. Since most of the studies described were performed using ATP levels of 100-200 μ M, a Mg:ATP ratio of 1:1 was consistently used.

pH Optimum Studies

The four Tdr kinase forms from both human normal liver and hepatoma tissues displayed unique pH optima. The bimodal pH activity curves for liver and hepatoma Tdr kinase IIB preparations, which showed optimum activity at pH's 7.5-8.0 and 9.0, are in agreement with the bimodal pH optima of 7.0 and 8.5 reported for human adult liver by Taylor *et al.* (1972). On the other hand the values for the hepatoma IIB preparation differ from the uniphasic pH optimum of pH 7.0 reported for human HeLa and KB cells, and human foetal liver (Taylor *et al.*, 1972) and for human adrenal glands and adrenocortical carcinomas (Nawata *et al.*, 1976). In the latter case the authors reported that the pH optima in normal and cancerous adrenal tissues were similar.

The existence in human normal liver of four Tdr kinases with pH optima ranging from pH 5.5 to 10.0 may explain the very broad pH optimum, in this range, found in the present study for a heterogeneous Tdr kinase preparation partially purified from adult human normal liver. Examination of Figure 20 reveals that the contribution of the individual Tdr kinase forms may account for the apparently broad pH optimum of this enzyme preparation.

The kinetic and stability studies described for the Tdr kinase forms from human normal liver and hepatoma tissues were performed at pH 6.5 to reduce the effects of contaminating ATPase activity. It can be seen from Figure 20 that at least 60% of the optimum activity of each

Tdr kinase form was measurable at this pH. The stability constant for Mg-ATP decreases markedly at lower pH's thus decreasing levels of Mg-ATP. This effect may account for the decreases in Tdr kinase activity at pH's below 5.5.

Thermostability Studies

With the exception of Tdr kinase IIA the corresponding forms of the enzyme from human normal and cancerous liver tissues showed similar heat stability. These findings differ from the findings that the human foetal liver and tumour cell form of Tdr kinase is more labile than the enzyme form from human adult liver (Taylor *et al.*, 1972; Stafford and Jones, 1972) and that Tdr kinase from human adrenal carcinomas is more labile to heat treatment than is the enzyme from normal adrenal gland (Nawata *et al.*, 1976). The Tdr kinase IIA preparation from human normal liver was the most stable of the enzyme forms from human normal and cancerous liver.

All of the Tdr kinase forms from human normal liver and hepatoma tissues were stabilised against heat inactivation by the presence of thymidine (100 μ M). Similar results have been obtained for Tdr kinases from calf thymus (Her and Mompalmer, 1971), rat regenerating liver (Wallockers and Wanka, 1974), Walker rat carcinosarcoma (Bresnick and Thompson, 1965), and virally-transformed HeLa cells (Cheng and Ostrander, 1976).

Ammonium Sulphate Inhibition

The inhibition by ammonium sulphate of human normal liver and hepatoma Tdr kinases is similar to that reported for virally-induced Tdr kinases in HeLa cells (Cheng and Ostrander, 1976), and that for mitochondrial and cytoplasmic forms of the enzyme from human leukaemic blast cells (Lee and Cheng, 1976b). These workers have shown that the marked

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