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Some enzymes of DNA synthesis in normal and cancerous human and rat livers

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inhibition caused by ammonium sulphate concentrations in the range 25-150μM appears to be due to the sulphate moiety, since sodium sulphate produced a similar effect whereas inhibition by sodium chloride was very low.

The finding of inhibition due to ammonium sulphate would account for the very low Tdr kinase activity noted during initial purification studies when ammonium sulphate fractionation techniques were used to isolate Tdr kinase. On the basis that a 100% saturated (70g/100ml) solution of ammonium sulphate has a concentration equivalent to 5.3M, it can be calculated that a protein solution which is 20% saturated with ammonium sulphate would have an ammonium sulphate concentration of 1M. Assuming that, after centrifuging down the precipitated protein, only 10% of the original ammonium sulphate concentration was present in the solution of redissolved protein, the data of Figure 23 show that an assay concentration of 50μM ammonium sulphate could inhibit the activity of all four of the Tdr kinase forms obtained from human normal liver or hepatoma tissues by about 75%.

Since approximately only 50% of the total Tdr kinase activity could be restored by desalting the enzyme preparation, a good proportion of the observed decrease in Tdr kinase activity probably represented permanent protein denaturation during ammonium sulphate fractionation.

Kinetic Constants for Thymidine

The Michaelis constants for thymidine were found to differ for the four Tdr kinase forms in human normal liver and hepatoma. The constants for the corresponding forms of Tdr kinase from these tissues were similar.

The Michaelis constants for thymidine obtained, in the present study, for the Tdr kinase preparations from human liver and hepatoma tissues were of the same order of magnitude as those reported for other mammalian Tdr kinases: 3.2μM for rat regenerating liver (Kizer and Holman, 1974); 2.1μM and 2.5μM respectively for adult and foetal rat liver (Klemperer and Haynes, 1969); 9μM for human laryngeal carcinoma tissue (Gordon *et al.*, 1968); and 2.6 and 5.2μM respectively for the cytoplasmic and mitochondrial forms of the enzyme from human leukaemic blast cells (Lee and Cheng, 1976c).

It should be noted that the literature K_m values were measured at pH 7.5 to 8.0 whereas those in the present study were measured at pH 6.5, to minimise possible effects due to ATPase contamination.

Due to the high ATPase contamination of most of the human normal liver and hepatoma Tdr kinase preparations, reliable K_m values for ATP could not be determined.

Phosphate Donor Studies

The effectiveness of the nucleotide triphosphates as phosphate donors decreased in the order:

ATP and CTP > GTP and dGTP and UTP > dATP and dCTP > dTTP.

CTP was equally as effective as ATP as a phosphate donor for the Tdr kinase forms from both human normal liver and hepatoma. These findings are in agreement with those reported for human adult liver Tdr kinase (Taylor *et al.*, 1972) and the mitochondrial form of the enzyme from human leukaemic blast cells (Lee and Cheng, 1976c). On the other hand, these findings differ from those obtained for the enzyme from human foetal liver, and HeLa and KB cell-lines (Taylor *et al.*, 1972) and calf thymus (Her and Momparler, 1971), where CTP was shown to be used to a lesser extent than ATP.

The low activity found with dCTP as a phosphate donor for the Tdr kinase forms from human normal liver and hepatoma tissues, is in agreement with that for the enzyme from human adult and foetal liver (Taylor *et al.*, 1972) and is similar to that for calf thymus Tdr kinase (Her and Momparler, 1971).

The finding that dATP was less effective than ATP as a phosphate donor for the Tdr kinases from human normal and cancerous liver differs from that for the enzyme from human adult and foetal livers (Taylor *et al.*, 1972), and that for the cytoplasmic form of Tdr kinase from human leukaemic blast cells (Lee and Cheng, 1976c). For the mitochondrial form of Tdr kinase from human leukaemic blast cells, however, the latter authors have shown that dATP is less effective as a *phosphate donor* than ATP.

The results for ATP and CTP, and dATP and dCTP suggest that the presence of a deoxyribose moiety renders these nucleotides less effective as phosphate donors for the human liver Tdr kinase preparations.

The low activities measured using GTP, dGTP, and UTP as phosphate donors for the human liver and hepatoma Tdr kinase forms are in agreement with those for the enzymes from human adult and foetal liver tissues (Taylor *et al.*, 1972) and calf thymus Tdr kinase (Her and Momparler, 1971). The higher activities obtained with GTP, dGTP, and UTP for the normal and cancerous liver Tdr kinase IB and IIA preparations *suggest* that these forms show a lower specificity for the phosphate donor than do the Tdr kinase IA and IIB *preparations*.

dTTP did not serve as a phosphate donor for the human liver or Tdr kinase forms, a finding which is in agreement with those for Tdr kinases from human adult and foetal liver (Taylor *et al.*, 1972), calf thymus (Her and Momparler, 1971), and the enzyme forms induced in HeLa TK^- cells by

Herpes simplex viruses Types I and II (Cheng, 1976).

In general the results of the phosphate donor studies using the human normal liver and hepatoma Tdr kinase preparations showed close agreement with those reported for the enzyme from human adult liver by Taylor *et al.* (1972). With respect to the phosphate donors studied, differences found for the corresponding Tdr kinase forms from human normal liver and hepatoma were small, and are probably not significant.

Nucleotide Inhibitor Studies

The phosphorylation of Tdr by ATP using Tdr kinase preparations from human normal liver and hepatoma showed little or no inhibition due to CTP, dUMP, dGTP or UTP. On the other hand, phosphorylation by these Tdr kinases was inhibited when dTMP, dTDP, dTTP, dATP or dCTP were included in the assay mixture. The nucleotides tested produced similar levels of inhibition of the Tdr kinase forms from both human normal liver and hepatoma tissues.

The inhibition for human liver and hepatoma Tdr kinases by dCTP and dATP is similar to that for the mitochondrial form of the enzyme from human leukaemic blast cells (Lee and Cheng, 1976c). On the other hand these authors have reported that the cytoplasmic form of the enzyme from human leukaemic blast cells is not inhibited by these nucleotides. The finding of inhibition by dCTP for the human normal and cancerous liver Tdr kinases is in agreement with the finding of inhibition by dCTP for human adult liver enzyme preparations (Taylor *et al.*, 1972), but differs from the absence of inhibition by dCTP found for Tdr kinases from human foetal liver, and HeLa or FB cell-lines (Taylor *et al.*, 1972), HeLa cells (Kit and Leung, 1974), and rat regenerating liver (Bresnick *et al.*, 1970). The finding of inhibition by dATP for human liver and hepatoma Tdr kinases differs from that of Taylor *et al.* (1972), who report no

inhibition due to this nucleotide for the human adult and foetal liver preparations. It may be argued that dCTP and dATP compete with ATP for the phosphate donor substrate-binding site, leading to decreased activity.

The finding of inhibition by dTMP, dTDP, and dTTP for human liver and hepatoma Tdr kinases, is in agreement with those for Tdr kinase preparations from human adult and foetal liver, and human cancerous cell-lines (Taylor *et al.*, 1972), and calf thymus (Her and Momparler, 1971).

It has been suggested that the Tdr kinases from human mammary, laryngeal, lung, and kidney tumours are less susceptible to inhibition by dTDP and dTTP than the enzyme forms from the corresponding normal tissues (Gordon *et al.*, 1968). In the present study the inhibitory effects of these nucleotides on the human liver and hepatoma Tdr kinase preparations appeared to be similar. This is in agreement with the finding that inhibition by dTTP for Tdr kinase from human normal adrenal glands is similar to that from adrenocortical carcinomas (Nawata *et al.*, 1976). The nature of the inhibition produced by the thymidine nucleotides is probably complex since, apart from possible effects on the phosphate donor binding site, dTDP and dTTP can also act as feedback inhibitors of Tdr kinase (Breitman, 1963; Ives *et al.*, 1963). Inhibition by dTTP has been shown to be competitive with respect to thymidine for the enzyme from rat regenerating liver (Breitman, 1963).

Inhibition by dUMP was tested to determine whether it could block the Tdr kinase salvage pathway, as it serves as the substrate for dTMP production in the *de novo* pathway of dTMP synthesis. The results obtained suggest that, for human liver and hepatoma Tdr kinase preparations, feedback inhibition due to this nucleotide does not occur.

The finding that UTP produced little or no inhibition of the human liver and hepatoma Tdr kinase forms is similar to the findings for the mitochondrial and cytoplasmic forms of Tdr kinase from human leukaemic blast cells (Lee and Cheng, 1976c), and the enzyme from mouse ascites sarcoma (Cheng and Prusoff, 1974).

The finding of the absence of inhibition by CTP for human normal and cancerous liver Tdr kinases is in agreement with the findings in this study (Table 20) that CTP is as efficient a phosphate donor as ATP. CTP has however been reported to produce slight inhibition of Tdr kinases from human adult and foetal liver (Taylor *et al.*, 1972), and human leukaemic blast cells (Lee and Cheng, 1976c).

The finding of low or absent inhibition by dGTP for human liver and hepatoma Tdr kinases is in agreement with the finding for the enzyme from human adult liver (Taylor *et al.*, 1972), but differs from the findings of these authors for the enzyme from human foetal liver, and Tdr kinases from the mitochondrial and cytoplasmic fractions of human leukaemic blast cells (Lee and Cheng, 1976c).

Thymidine Analogue Studies

Thymidine analogue studies using a crude preparation from human normal liver, which contained all four Tdr kinase forms, suggested that, relative to thymidine: deoxyuridine, 5-fluoro-deoxyuridine, and deoxycytidine were effective phosphate acceptors; cytidine, 5-iodo-deoxyuridine, and 5-bromo-deoxyuridine were less effective phosphate acceptors; and thymine, uracil, uridine, and 5-fluoro-uridine showed little or no ability to act as phosphate acceptors.

The findings for deoxyuridine (Udr) and the 5-halo-derivatives of Udr (5-F-Udr, 5-Br-Udr and 5-I-Udr) are similar to those for Tdr kinases from rat hepatomas and sarcomas (Bresnick and Thompson, 1965) and calf

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thymus (Her and Momparler, 1971). The finding that deoxycytidine and to a lesser extent cytidine served as phosphate acceptors, differs from the findings for Tdr kinase from Yoshida sarcomas (Hashimoto *et al.*, 1972). Because of the non-specificity of the ^{32}P-ATP assay used in these studies, the possibility cannot be excluded that the effects observed with deoxycytidine and cytidine may have been due to the presence of contaminating enzymes such as uridine-cytidine kinase or deoxycytidine kinase. However, uridine-cytidine kinase from Ehrlich ascites tumour cells has been shown to be specific for uridine or cytidine (Skold, 1960); and calf thymus deoxycytidine kinase is specific for deoxycytidine (Durham and Ives, 1970; Ives and Durham, 1970). Attempts to explain the results of the present study as being due to contamination by these enzymes would require the presence of both these contaminating enzymes in the liver Tdr kinase preparation, in which case there would not be an explanation for the failure of uridine to be phosphorylated by a uridine-cytidine kinase.

The ability of the thymidine analogues to inhibit the phosphorylation of thymidine was tested using the more highly purified Tdr kinase preparations from human normal liver and hepatoma. It was found that deoxycytidine, 5-I-Udr, 5-Br-Udr, and 5-F-Udr inhibited the Tdr kinases from both human normal and cancerous liver, when present at concentrations five-fold greater than those of the saturating Tdr levels. An exception was found in the case of the hepatoma Tdr K IA preparation, which was apparently activated in the presence of 5-F-Udr. The bases thymine and uracil inhibited the liver and hepatoma Tdr K IA forms, but produced little or no inhibition of the other Tdr kinase preparations. Cytidine, uridine, and Udr produced little or no inhibition of the human normal liver and hepatoma Tdr kinases.

The poor inhibition by cytidine, when compared with the marked inhibition produced by the presence of deoxycytidine, suggests that the Tdr kinase enzyme forms show some specificity for deoxyribonucleosides. This is supported by the finding that deoxycytidine, deoxyuridine, and 5-fluoro-deoxyuridine were more effective as substrates than were cytidine, uridine, and 5-fluoro-uridine.

Deoxyuridine did not inhibit the phosphorylation of Tdr by human liver and hepatoma Tdr kinase preparations, although the analogue studies suggested that it was an effective phosphate acceptor. Similarly, it has been shown that Udr does not affect Tdr phosphorylation catalysed by mitochondrial or cytoplasmic Tdr kinase forms from human leukaemic blast cells (Lee and Cheng, 1976c). Lee and Cheng have demonstrated that the inhibition constants (K_i's) for Udr for these enzyme preparations are two orders of magnitude greater than the K_m's for Tdr. This may explain the failure of Udr to inhibit Tdr kinase activity in the present studies. On the other hand, the ability of Udr to act as a phosphate acceptor may have been due to the presence of a contaminating Udr kinase, in which case, when using the more specific monitoring of the conversion of ^{3}H-Tdr to ^{3}H-dTMP, no inhibitory effect on Tdr kinase would be expected.

Comparison of the inhibitory effects of the halo-Udr derivatives shows that, in general, the 5-bromo- and 5-iodo-derivatives produced greater inhibition than 5-F-Udr. These findings are similar to those for Tdr kinases from human leukaemic blast cells (Lee and Cheng, 1976c). Tdr kinases from rat carcinosarcomas have also been shown to be inhibited by 5-F-Udr, 5-Br-Udr, and 5-I-Udr (Bresnick and Thompson, 1965). A possible explanation for the less marked inhibition due to 5-F-Udr is the finding by Lee and Cheng (1976c) that the K_i for 5-F-Udr, for the Tdr kinases from human leukaemic blast cells, is at least an order of

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magnitude greater than those for 5-Br-Udr and 5-I-Udr. There is at this stage no explanation for the apparent activation by 5-F-Udr of the human hepatoma Tdr K IA preparation.

The present study suggests that, with the exception of the differential effect of 5-F-Udr on the Tdr K IA preparations, the inhibitory effects due to Tdr analogues on the corresponding forms of the enzyme from human normal liver and hepatoma tissues are not significantly different.

CONCLUSIONS

Four forms of Tdr kinase were purified from both human normal liver and hepatoma. The corresponding Tdr kinase forms in human normal liver and hepatoma were shown to be similar with respect to their proportions in the normal and cancerous liver tissues and, in general, with respect to their physical and kinetic properties. Additional Tdr kinase forms, as described in human foetal liver and in some human tumour cell-lines (Taylor *et al.*, 1972), were not present in the human hepatoma tissues from which Tdr kinases were purified in the present study.

5-Fluoro-uracil and its derivatives, such as 5-F-Udr, are widely used in cancer chemotherapy as pyrimidine antagonists. The drug has been shown to be active against a wide range of solid tumours, and significant responses to therapy have been reported in human cancers of the large bowel, liver, breast, stomach, ovary, thyroid, pancreas, cervix, pharynx, and urinary bladder (Dowling *et al.*, 1970; Oates, 1972). 5-F-Udr may be converted to 5-F-dUMP by Tdr kinase and the 5-F-dUMP thus formed will inhibit thymidylate synthetase and thus block synthesis of dTTP and DNA by the *de novo* pathway (page 12). The results of the present study suggest that 5-fluoro-deoxyuridine can be used as a substrate by both human normal liver and hepatoma Tdr kinases. The finding that Tdr could

be converted to dTMP in spite of the presence of high levels of 5-F-Udr suggests that the Tdr kinase salvage pathway may still operate in liver cells of patients receiving 5-F-Udr chemotherapy. This may explain the finding by Barret and Cohen (1968) that 5-F-Udr did not block dTTP synthesis in human regenerating liver following surgical removal of a hepatoma.

SUMMARY

1. Thymidine kinases from human normal liver and hepatoma tissues were purified, and some of their physical and kinetic properties were studied.

2. Four forms of the enzyme were separated from both human normal liver and hepatoma. The forms were designated thymidine kinases IA, IB, IIA, and IIB.

3. The proportions of each of the forms in normal liver were shown to be similar to the corresponding forms in hepatoma. The IIB form of the enzyme was the predominant form in both human liver and hepatoma.

4. The molecular weights of the liver and hepatoma enzyme forms were: IA 130 000; IB 105 000; IIA > 1 x 10^6; and IIB 80 000.

5. A Mg-ATP ratio of 1:1 gave optimum enzyme activity at concentrations of ATP greater than 50µM, whereas this ratio increased to 5:1 at lower ATP levels.

6. The pH optima for the liver and hepatoma enzyme forms were: IA 6.0 - 6.5; IB 7.5 - 8.0; IIA 7.5 - 8.0; and IIB 7.0 - 8.0 and 9.0 (biphasic).

7. All the enzyme forms lost activity on heating at 55°C for five minutes. This loss of activity could be significantly reduced in the presence of 100µM thymidine.

8. Ammonium sulphate at concentrations greater than 20mM produced significant inhibition of all four forms of the enzyme.

9. The apparent Michaelis constants for Tdr for the enzyme forms from normal and cancerous liver, respectively, were:

IA, 15.5 and 17.3µM; IB 8.4 and 6.9µM; IIA, 4.8µM and 4.8µM; IIB, 4.9 and 4.5µM.

10. The hepatoma and liver enzyme forms utilised ATP and CTP as phosphate donors, and to a lesser extent GTP, dGTP, and UTP. dATP, dCTP, and dTTP showed little or no ability to serve as phosphate donors.

11. The hepatoma and liver forms of the enzyme were inhibited by dCTP, dTMP, dTDP, dTTP, and dATP whereas CTP, dUMP, dGTP, and UTP had little or no inhibitory effect.

12. Studies on the ability of thymidine analogues to act as phosphate acceptors showed that deoxyuridine, 5-fluoro-deoxyuridine, and deoxycytidine were as effective as thymidine, whereas cytidine, 5-bromo-deoxyuridine, and 5-iodo-deoxyuridine were less effective than thymidine; and thymine, uracil, uridine, and 5-fluoro-uridine showed little or no phosphorylation.

13. Thymidine phosphorylation by the thymidine kinase forms from human normal and cancerous liver was inhibited by the thymidine analogues 5-fluoro-deoxyuridine, 5-bromo-deoxyuridine, 5-iodo-deoxyuridine, and deoxycytidine; with the exception that phosphorylation by the hepatoma thymidine kinase IA preparation was activated by 5-fluoro-deoxyuridine.

14. In general the physical and kinetic properties of the corresponding thymidine kinase forms from human liver and hepatoma were comparable.

Abbreviations

ADP	adenosine 5'-diphosphate
Ara-CTP	cytosine arabinoside 5'-triphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase [ATP phosphohydrolase (EC 3.6.1.3)]
5-Br-Udr	5-brome-deoxyuridine
c-AMP	adenosine-3'-5'-cylic monophosphate
CDP	cytidine 5'-diphosphate
Cdr	deoxycytidine
Ci	Curie
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCDP	2'-deoxycytidine 5'-diphosphate
dCMP	2'-deoxycytidine 5'-monophosphate
dCMP deaminase	deoxycytidylate deaminase [dCMP aminohydrolase (EC 3.5.4.12)]
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNA polymerase	deoxyribonucleic acid polymerase [deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7)]
dTDP	2'-deoxythymidine 5'-diphosphate
dTMP	2'-deoxythymidine 5'-monophosphate
dTMPase	* thymidylate 5'-phosphatase [thymidylate 5'-phosphohydrolase (EC 3.1.3.35)]
dTMP kinase	* thymidylate kinase [ATP:deoxythymidine-monophosphate phosphotransferase (EC 2.7.4.9)]

dTMP synthetase	* thymidylate synthetase [N^5,N^{10}-methylene tetrahydrofolate:2'-deoxyuridylate C-methyltransferase (EC 2.1.1.-)]
dTTP	2'-deoxythymidine 5'-triphosphate
5-F-Udr	5-fluoro-2'-deoxyuridine
5-F-dUMP	5-fluoro-2'-deoxyuridine 5'-monophosphate
GTP	guanosine 5'-triphosphate
5-I-Udr	5-iodo-2'-deoxyuridine
K_m	Michaelis constant
3' Me-DAB	3'-methyl-4-dimethylaminoazobenzene
5-Me-dCMP	5-methyl-2'-deoxycytidine 5'-monophosphate
N^5,N^{10}-Me-THFA	N^5,N^{10}-methylene-tetrahydrofolic acid
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced from
OMP	orotidine 5'-monophosphate
PLC	primary liver cancer
R_f	migration relative to the solvent front in chromatography
R_m	migration relative to the marker front in electrophoresis
RNA	ribonucleic acid
Tdr	*thymidine
Tdr kinase (or Tdr K)	*thymidine kinase [ATP:thymidine 5'-phosphotransferase (EC 2.7.1.75)]
THFA	tetrahydrofolic acid
UDP	uridine 5'-diphosphate
Udr	2'-deoxyuridine
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate

* The prefix 'deoxy-' is, by convention, frequently omitted when describing deoxythymidine and its derivatives, since all thymine derivatives are found only as deoxyribonucleosides and deoxyribonucleotides.

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