STUDIES ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID BY EXTRACTS OF MAMMALIAN CELLS

V. FACTORS INTERFERING WITH BIOSYNTHESIS

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SUMMARY

Cell free extracts of certain tissues such as Ehrlich ascites carcinoma, regenerating rat liver and rabbit bone marrow contain kinases which promote the conversion of thymidine to the mono- di- and triphosphates and an enzyme which catalyses the polymerization of the four deoxyribucleoside triphosphates into DNA.

Such enzymes are much less abundant in extracts of non-proliferating tissues such as liver and kidney.

The addition of extracts of liver or kidney greatly reduces the ability of extracts of ascites cells, bone marrow or regenerating rat liver to phosphorylate thymidine and to form DNA.

Extracts of normal liver and of ascites cells contain kinases which bring about the phosphorylation of the monophosphates of deoxyadenosine, deoxyguanosine, and deoxycytidine. The addition of liver extracts to ascites extracts causes no reduction in kinase activity in such systems.

INTRODUCTION

In previous communications [1–4] we have reported the presence in cell free extracts of Ehrlich ascites carcinoma cells and other mammalian tissues, of kinases which bring about the phosphorylation of TdR to TTP and of an enzyme which promotes the polymerization of the four deoxyribonucleoside triphosphates to form DNA in a manner similar to that described by KORNBERG *et al.*[5] for *E. coli*. Such enzymes are also present in extracts of regenerating rat liver[6–9].

The present paper describes the effects of extracts of normal liver tissue on the kinases and polymerases from other tissues. A preliminary report has already appeared[10].

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dAMP, dGMP, dCMP and TMP, 5′-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively; dATP, dADP, dGDP, dGTP, dCTP, dCDP, TTP and TDP, 5′-tri- and diphosphates respectively of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; TdR, thymidine; ATP, adenosine-5′-triphosphate; DPN, diphosphopyridine nucleotide; DNase, deoxyribonuclease; RNase, ribonuclease; TCA, trichloroacetic acid; Tris, 2-amino-2-hydroxymethyl propane-1, 3-diol.

METHODS

Biological

The Ehrlich ascites carinoma, kindly supplied by Dr. G. POPJÁK of Hammersmith Hospital, London, was maintained by serial transplantation in mice of the departmental colony. Rabbits weighing approximately 2000 g and rats of about 200 g were obtained from the departmental colonies. Partial hepatectomies were kindly performed by Dr. R. Y. THOMSON by the procedure of HIGGINS AND ANDERSON[11].

Preparation of tissue extracts

All manipulations were carried out in the cold. Extracts of Ehrlich ascites tumour cells were prepared from osmotically disrupted cells by centrifuging at 105,000 × *g* as previously described[1]. Extracts of rabbit tissues were prepared by homogenising in 10 volumes distilled water or 0.1 *M* Tris buffer pH 7.9 for 2 min in a Potter type of homogeniser. Rat livers were homogenised in 20 ml distilled water for 2 min in a Potter type homogeniser. Both types of homogenate were centrifuged at 105,000 × *g* for 1 h and the supernatant fluid decanted.

In some experiments liver extracts or fractions prepared from them were heated at 100° for 5 min and centrifuged for 10 min at 600 × *g* to remove precipitated protein.

Dialysis of tissue extracts or fractions was carried out for varying times against 0.1 *M* Tris buffer pH 7.9, 0.001 *M* with respect to 2-mercaptoethanol.

Preparation of deoxyribonucleoside 5'-triphosphates

Some samples of dATP and dGTP were prepared by a modification of the biological method of KEIR AND SMELLIE[2] as follows: A reaction mixture was set up containing the following components in a total volume of 120 ml: 300 μmoles dAMP (sodium salt), 900 μmoles ATP (sodium salt), 10 mmoles Tris buffer pH 7.9, 900 μmoles $MgCl_2$, 400 mg lyophilised Ehrlich ascites extract. The mixture was incubated at 37° for 7 h with shaking and was then heated to 100° for 3 min and rapidly cooled to 0°. The precipitate of protein was removed by filtration through Whatman No. 1 paper at 0°. Samples of the filtrate were taken for paper chromatography in the ammonium isobutyrate system[2] to obtain a measure of the extent of conversion to the triphosphate. The bulk of the solution was diluted to 2 l with distilled water and applied to a column of Dowex-1-Cl, 31 cm × 2 cm. After washing with water the column was eluted successively with 0.003 *N* HCl, 0.01 *N* HCl–0.02 *M* LiCl and 0.01 *N* HCl–0.2 *M* LiCl to bring off peaks corresponding to AMP + dAMP, ADP + dADP and ATP + dATP respectively. The tubes corresponding to ATP + dATP were pooled, neutralised with LiOH and concentrated to small volume in an Edwards Model 10P freeze drier. An equal volume of methanol and 20 volumes of acetone were added and the mixture cooled to −10° for 1 h to precipitate the lithium salts of ATP and dATP. These were collected by centrifugation and dried in air. The lithium salts were dissolved in distilled water and a sample taken for chromatography in the ammonium isobutyrate system to check that only ATP and dATP were present. The main bulk of the solution was made up to 55 ml and half of it taken for periodate oxidation to destroy ATP. 11.8 ml 0.1 *M* glycylglycine buffer pH 7.4 were added followed by 900 μmoles $NaIO_4$ and the mixture was incubated at 25° for 30 min

with frequent shaking. Excess periodate was removed by adding 1.2 mmoles glucose followed by incubation for 30 min at 25°. The pH was adjusted to 10 by the addition of 10.4 ml *M* glycine buffer pH 10.2 and the solution incubated for 14 h at 37°. The alkaline incubation mixture was diluted to 350 ml with water and applied to a Dowex-1-Cl column, 13.5 cm × 2 cm. The column was washed with water (80 ml) and elution was carried out with 0.01 *N* HCl–0.02 *M* LiCl, 0.01 *N* HCl–0.08 *M* LiCl and 0.01 *N* HCl–0.20 *M* LiCl. This last solvent brought off the dATP and the tubes containing this were pooled, neutralised with LiOH, and the lithium salt precipitated with methanol and acetone after reduction of the volume. The lithium salt was dried in air, dissolved in water, a sample taken for chromatography and the remainder converted to the sodium salt by passage through a Dowex-50-Na column. The other half of the mixture of lithium salts of ATP and dATP was treated in the same manner and the products pooled. Paper chromatography revealed only one major spot corresponding to dATP and having a phosphorus:deoxyadenosine ratio[2] of 2.98:1. The total yield of dATP by this procedure was 200 μmoles.

Preparations of dGTP were made in the same manner except that it was not necessary to carry out the periodate oxidation to remove ATP since dGTP and ATP can be separated on Dowex-1-Cl columns. All the nucleotides except dGTP were eluted with 0.1 *N* HCl–0.1 *M* LiCl and the dGTP was eluted with 0.01 *N* HCl–0.2 *M* LiCl. The ratio of phosphorus to deoxyguanosine in the final product was 2.97.1 and on paper chromatography in ammonium isobutyrate it migrated as a single spot.

Other specimens of dATP and dGTP were prepared by a slight modification of the method of SMITH AND KHORANA[12] from dAMP and dGMP. dCTP and TTP were also prepared chemically from dCMP and TMP respectively by the SMITH AND KHORANA[12] procedure. TDP was obtained as a by-product of TTP synthesis.

TTP labelled in the proximal position with ^{32}P (TdR-^{32}P-P-P) was prepared from [^{32}P]TMP by the same method. For the preparation of [^{3}H]TTP, [^{3}H]TMP prepared as already described[4], by the WILZBACH procedure, was diluted with 10 parts of non-radioactive TMP and phosphorylated by the SMITH AND KHORANA[12] method.

[^{32}P]TMP was prepared from 500 μmoles TdR, 100 mC carrier free $^{32}PO_4$ and 225 mg 85 % orthophosphoric acid by the method of HURWITZ[13]. When the initial reaction was complete, the [^{32}P]TMP and unreacted TdR were separated from inorganic ortho- and pyrophosphate by adsorption on a charcoal column, 10 cm × 3.5 cm. The column was washed with water and 0.01 *M* $NaHCO_3$ (see ref. 14) until the radioactivity of the eluate dropped to a low level. The TdR and TMP were then eluted with 70 % aqueous ethanol 0.14 *M* with respect to ammonia. The eluate was concentrated *in vacuo* at 37° and the concentrate applied to a Dowex-1-Cl column. The column was washed with water to remove all traces of TdR and the TMP was eluted with 0.05 *N* HCl. The eluate was concentrated *in vacuo* at 37° and then lyophilised. Paper chromatography of the TMP showed that it consisted essentially of [^{32}P]TMP with a little contaminating [^{32}P]-inorganic orthophosphate but no thymidine, pyrophosphate or TMP-3′. The overall yield was 108 μmoles TMP. This TMP was used directly as previously described for the preparation of [^{32}P]TTP. The specific activity of the product was approximately $2 \cdot 10^6$ counts/min/*μmole* as determined in a windowless gas flow counter.

Other samples of [^{32}P]TMP were prepared by the method of TENER[15] from TdR and [^{32}P]cyanoethylphosphate. [^{32}P]dAMP, [^{32}P]dGMP and [^{32}P]dCMP were also

prepared in this way. The specific activity of all these nucleotides was 0.5–2.0·10^6 counts/min/μmole.

DNA for use as primer was obtained from the 105,000 × *g* sediment of the osmotically disrupted Ehrlich ascites cells by the detergent procedure of KAY, SIMMONDS AND DOUNCE[16].

Fractionation of Ehrlich ascites cell extracts

Extracts of Ehrlich ascites cells prepared as described above were adjusted to pH 4.5 in the cold by the addition of 1 *N* acetic acid. The resulting suspension was centrifuged at 600 × *g* for 10 min and the clear supernatant was decanted into a separate vessel and neutralised with 1 *N* NaOH (fraction AS 4.5). This fraction contains the larger part of the kinase activities and was generally used as a source of enzyme in inhibition tests. The sediment was dispersed in ice-cold distilled water to give a protein concentration of 6–8 mg/ml and the pH was adjusted to 7.0–7.5 by the addition of 0.1 *N* NaOH (fraction AP 4.5). This fraction contains the bulk of the polymerase activity and was generally used as a test material for polymerase inhibition.

Fractionation of liver extracts

Extracts of normal and regenerating liver were fractionated by the serial addition of solid ammonium sulphate. The precipitates were centrifuged down and dissolved in a volume of water equal to the original volume of extract.

Assay of kinases

Thymidine kinase (the enzyme catalysing the formation of TMP from TdR), TMP kinase (the enzyme responsible for the formation of TDP from TMP), and TDP kinase (the enzyme responsible for the formation of TTP from TDP) were assayed as described previously[4].

Kinases responsible for the formation of dATP, dGTP and dCTP from dAMP, dGMP and dCMP were assayed as follows:

The tissue extracts were incubated for 90 min at 37° in Tris buffer pH 7.4 in the presence of KCl, $MgCl_2$ ATP and the appropriate substrate. The tubes were chilled in ice and treated with 2 *N* perchloric acid to a final concentration of 0.6 *N*.

The precipitated protein was centrifuged down and the supernatant fluid neutralised with KOH. The precipitated $KClO_4$ was removed by centrifugation and the supernatant fluid either stored in the frozen state or processed immediately.

In each experiment a non-incubated control was set up in which protein was precipitated with perchloric acid before addition of substrate.

The neutral extract was diluted to about 20 ml and applied to a Dowex-1-Cl column (6.5 × 1.5 cm). The column was washed with 20 ml water and eluted with 100-ml portions of dilute acid according to the following scheme:

[^{32}P]dCMP	1. 0.01 *N* HCl to remove monophosphates
	2. 0.01 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates
[^{32}P]dAMP	1. 0.01 *N* HCl to remove monophosphates
	2. 0.01 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates
[^{32}P]dGMP	1. 0.05 *N* HCl to remove monophosphates
	2. 0.02 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates

The eluates were appropriately diluted for counting in an M6 liquid counter attached to a conventional scaling unit.

Polymerase assay

The enzyme responsible for the formation of polynucleotide material from dATP, dGTP, dCTP and TTP was assayed by two procedures:

Net synthesis assay: An incubation was set up containing the enzyme preparation. 100 μmole Tris buffer pH 7.9, 0.25 μmole DPN, 5 μmoles $MgCl_2$, 25 μg DNA primer previously heated to 100° for 10 min and 300 mμmoles each of dATP, dGTP, dCTP and TTP in a volume of 1 ml. Incubation was carried out at 37° in centrifuge tubes with shaking for 2 h. In this assay all tests were carried out in triplicate or quadruplicate and an equivalent number of controls to which no deoxyribonucleoside triphosphates had been added was set up. The reaction was stopped by immersing the tubes in a mixture of ethanol and solid CO_2 and the material was either processed immediately or stored at —20° until required. The frozen incubation mixtures were allowed to thaw at 0° and, where the amount of enzyme protein was very low, 0.2 ml of 1 % solution of bovine serum albumin was added to facilitate precipitation. 0.5 volume ice-cold 2.1 *N* $HClO_4$ was added to precipitate protein and nucleic acid and the mixture was centrifuged. The supernatant fluid was decanted and the precipitate was washed twice more with cold 0.7 *N* $HClO_4$ to remove soluble nucleotides. The washed precipitate was dissolved in 2 ml 0.3 *N* KOH and two 0.8-ml portions of the solution were taken for the estimation of DNA by the CERIOTTI[17] procedure. The difference in DNA content between control and test incubations was taken as a measure of new polynucleotide synthesis.

The incorporation of [^{32}P]TTP: Reaction mixtures were set up containing 25 μmoles Tris buffer pH 7.9, 62.5 mμmoles DPN, 1.25 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 50 μg DNA (previously heated for 10 min at 100° and cooled quickly) and, generally, 50 mμmoles each of dATP, dGTP, dCTP and [^{32}P]TTP. The polymerase preparation was added, the total volume adjusted to 0.25 ml with water and the tubes incubated at 37° for 2 h in a shaking incubator. After incubation, the amount of radioactivity in the DNA was determined by the method described by BOLLUM[18]. 50-μl aliquots of the incubation mixtures were pipetted on to numbered discs of Whatman No. 1 filter paper, 1 in in diameter. The discs were dropped into a beaker of cold 5 % (w/v) TCA containing 15 ml TCA per disc and 5–10 min after the last disc had been added the batch was washed by swirling in the beaker. The TCA was removed by suction and replaced by fresh TCA and the whole process repeated twice more. Finally the discs were washed with 95 % ethanol, removed, dried, and counted in a Nuclear Chicago windowless gas flow counter. The above procedure was found to remove all radioactivity from non-incubated control discs. This assay has been shown to be reliable by comparison with the net synthesis method.

For studies on inhibitory factors, 0.075 ml of polymerase preparation was incubated with an equal volume of solution of potential inhibitor.

MATERIALS

ATP, dAMP, dGMP, dCMP, TMP, TdR, deoxyadenosine, deoxyguanosine, deoxycytidine and DPN were purchased from the Sigma Chemical Corporation. Some specimens

of ATP and DPN were purchased from C. F. Boehringer and of dAMP, dGMP, dCMP and TMP from Schwarz Bioresearch Inc. and from the California Corporation. [^{3}H]-TdR of specific activity 360 mC/mmole or 1.9 C/mmole was purchased from Schwarz Bioresearch Inc.

RESULTS

The results obtained with AS and AP from the supernatant fraction from Ehrlich ascites cells (Table I) show that such extracts contain an active polymerase and kinases capable of converting 75 % of the added TdR to TTP.

In extracts of normal rat liver, polymerase activity is low (Table I) but is increased in regenerating rat liver 48 h after partial hepatectomy. However, the addition of normal rat liver extracts to regenerating rat liver extracts greatly reduces the polymerase activity (Table I). The same effect is observed when rat liver extract is added to an ascites preparation (Table I).

Extracts of rabbit liver also show a low polymerase activity whereas extracts of bone marrow give a high value (Table I). Addition of liver extract to bone marrow extract reduces the polymerase activity to a very low level (Table I).

A similar picture is obtained with kinase activity (Table I) which is higher in extracts of regenerating liver, bone marrow and ascites tumour than in extracts of normal liver. Again, addition of extracts of normal liver to extracts of regenerating liver, bone marrow or ascites tumour reduces kinase activity as measured by the amount of TdR converted to phosphorylated derivatives (Table I).

TABLE I

POLYMERASE AND KINASE ACTIVITY IN EXTRACTS OF DIFFERENT TISSUES

Polymerase assay: Reaction mixtures contained 25 μmoles Tris buffer pH 7.9, 62.5 μmmoles DPN, 1.25 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 50 μg DNA (previously heated for 10 min at 100° and cooled quickly), generally 50 mμmoles each of dATP, dGTP, dCTP and [^{32}P]TTP and 0.075 ml of the polymerase preparation, all in a total volume of 0.25 ml. The mixtures were incubated with shaking in 10 × 1 cm test tubes for 2 h at 37°.
Kinase assay: Reaction mixtures contained 5.5 μmoles $MgCl_2$, 5.5 μmoles ATP, 110 μmoles Tris buffer pH 7.9, 0.5 μC[^{3}H]TdR and 0.35 ml of kinase preparation, all in a total volume of 1.1 ml. The mixtures were incubated with shaking in 25-ml conical flasks for 90 min at 37°. For inhibition effects, incubation mixtures contained equal volumes of enzyme and inhibitor preparations. These were 0.075 ml and 0.35 ml for polymerase and kinase respectively.
For liver and bone marrow assays whole extracts were employed.
For ascites cells, fraction AS 4.5 was used for kinase assays and fraction AP 4.5 for polymerase assays.

Tissue	*Polymerase**	*Kinases***		
		TMP	*TDP*	*TTP*
Ascites tumour	4.7	430	830	2130
Ascites tumour + rat liver	0.4	14	12	0
Rat liver	1.2	10	3	1
Regenerating rat liver	7.3	30	70	50
Normal + regenerating rat liver	0.6	40	20	7
Rabbit liver	0.4	10	2	1
Rabbit bone marrow	5.3	20	80	130
Rabbit liver + bone marrow	0.2	20	20	20

* mμmoles [^{32}P]TTP incorporated in 2 h /mg protein.
** $\mu\mu$moles formed in 1.5 h /mg protein.

TABLE II

KINASE ACTIVITIES IN EXTRACTS OF EHRLICH ASCITES CELLS AND OF LIVER TISSUE

The reaction mixture (1 ml) contained 50 μmoles Tris buffer pH 7.4, 80 μmoles KCl, 25 μmoles $MgCl_2$, 25 μmoles ATP (Na salt), 5 μmoles substrate, and two of the following —0.2 ml ascites extract, 0.2 ml liver extract, 0.2 ml water. Incubation time 90 min. Temperature, 37°.

Substrate	*Material tested*	*Total*	*% applied counts recovered*	
			Monophosphate	*Diphosphate + Triphosphate*
[^{32}P]dCMP	Control	100	95	5
	Ascites	85	69	16
	Liver	85	65	20
	Ascites + liver	87	59	28
[^{32}P]dAMP	Control	100	95	5
	Ascites	87	63	18
	Liver	85	60	25
	Ascites + liver	84	48	36
[^{32}P]dGMP	Control	100	91	9
	Ascites	82	64	18
	Liver	83	61	22
	Ascites + liver	75	39	36

It should be noted that the pattern of results obtained with the kinases for dAMP, dGMP and dCMP is different from that found for the kinases of thymine derivatives. Table II shows appreciable levels of these kinases in extracts of normal liver and ascites tumour cells. The addition of extracts of normal liver to ascites cell extracts causes no inhibition of kinase activity and even results in an additive effect. Moreover the difference between extracts of normal and of regenerating liver is but slight with these substrates (Table III), although the percentage of counts recovered is consistently greater for regenerating than for normal liver.

Although normal liver extracts show low polymerase activity, they nevertheless

TABLE III

KINASE ACTIVITIES IN EXTRACTS OF NORMAL AND REGENERATING RAT LIVER*

The conditions were as described in Table II. The reaction mixture (1 ml) contained 0.4 ml liver extract.

Substrate	*Material tested*	*Total*	*% applied counts recovered*	
			Monophosphate	*Diphosphate + Triphosphate*
[^{32}P]dCMP	Control	100	92	8
	Normal liver	88	59	29
	Regenerating liver	96	65	31
[^{32}P]dAMP	Control	100	93	7
	Normal liver	65	21	48
	Regenerating liver	89	39	50
[^{32}P]dGMP	Control	100	89	11
	Normal liver	62	36	26
	Regenerating liver	88	51	37

* 48 h after partial hepatectomy.

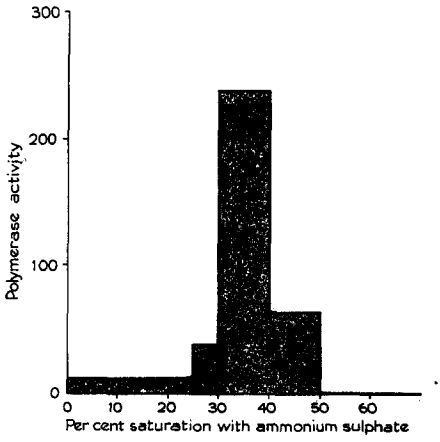

Fig. 1. Polymerase activity in fractions of normal liver extract prepared by ammonium sulphate precipitation. Enzyme activity was assayed as described in the text using 0.075 ml of each fraction after dialysis overnight against 0.1 *M* Tris buffer 0.001 *M* with respect to mercaptoethanol. Activity is expressed in terms of total counts/min of acid-precipitable radioactivity per reaction tube.

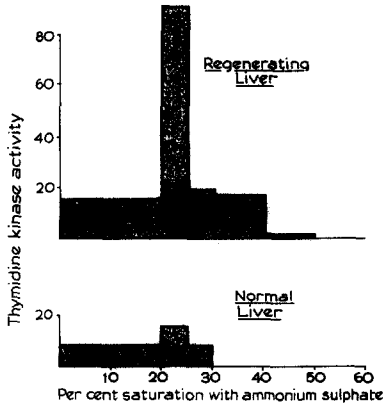

Fig. 2. Thymidine kinase activity in fractions of normal and regenerating rat-liver extracts prepared by ammonium sulphate precipitation. Enzyme activity was assayed as described in the text using 0.35 ml of a 1:5 dilution of the ammonium sulphate fractions, which had been dialysed overnight against 0.1 *M* Tris buffer 0.001 *M* with respect to mercaptoethanol. Activity is expressed as % conversion to TMP.

TABLE IV

% INHIBITION OF EHRLICH ASCITES POLYMERASE AND KINASES BY EXTRACTS OF VARIOUS TISSUES

The conditions were as described in Table I.

Tissue	*Polymerase*	*Phosphorylation to*	
		TMP + TDP + TTP	*TDP + TTP*
Rat liver	64	62	87
Rabbit liver	60	51	65
Rabbit kidney	92	0	39
Rabbit brain	0	0	0
Rabbit muscle	0	4	11
Calf serum	64	0	46
Bovine albumin	8	0	0

TABLE V

INHIBITION OF RABBIT TISSUE POLYMERASES BY LIVER AND KIDNEY EXTRACTS

Enzyme activity was assayed as described in Table I using 0.075 ml of polymerase preparation and an equal volume of liver or kidney extract.

Polymerase source	*% inhibition by*	
	Liver	*Kidney*
Spleen	23	85
Thymus	53	95
Bone marrow	85	95

contain an active enzyme which is concentrated mainly in the fraction precipitated between 30 and 40 % saturation with ammonium sulphate (Fig. 1).

The TdR kinase activity is concentrated in the fraction precipitated by ammonium sulphate between 20 and 25 % saturation and is obviously much higher in regenerating liver (Fig. 2). Such a fraction from normal liver phosphorylates 3 to 4 times as much TdR as does whole extract. No further increase in activity is obtained on subsequent fractionation with ECTEOLA columns[4].

The presence of interfering factors towards the polymerase and thymidine kinases of ascites tumour cells is shown in Table IV. Factors inhibiting polymerase are pronounced in liver, kidney and serum but not in brain, muscle or bovine serum albumin. The phosphorylation of thymidine is strongly inhibited by liver extracts but not by extracts of the other tissues studies while the formation of TDP and TTP is inhibited by liver extracts and by extracts of kidney, by serum and to a much less pronounced extent by muscle extracts (Table IV).

Similar inhibition of polymerase activity from some other sources by extracts of liver and kidney is shown in Table V.

No inhibition of polymerase or kinase activity was observed with boiled liver extract, with the acid-soluble fraction prepared from liver extract by removal of protein with acid, or with dialysates of liver extract.

DISCUSSION

The results in Table I show that extracts of liver and ascites cells are capable of phosphorylating dCMP, dGMP and dAMP. This is in agreement with the results of HECHT, POTTER AND HERBERT[19] for dCMP with normal liver homogenates and of SABLE *et al.*[20] for dAMP with rabbit kidney mitochrondria. It is clear, however, from Table I that the addition of liver extract to ascites extract results in no inhibition of phosphorylation and indeed shows an additive effect.

Extracts of regenerating liver also phosphorylate dCMP, dGMP and dAMP (Table III) in agreement with the results of CANELLAKIS AND MANTSAVINOS[8,21] and of BOLLUM AND POTTER[6]. Our results show that there is no great difference between extracts of normal and of regenerating liver.

The position as regards the phosphorylation of thymidine and thymidine nucleotides is quite different (Table I). Extracts of rapidly proliferating cells such as ascites tumour, regenerating rat liver and rabbit bone marrow catalyse extensive formation of TMP, TDP and TTP from thymidine whereas extracts of non-proliferating tissues[4] such as normal liver have little activity. The development of enzymes for the phosphorylation of thymidine derivatives in regenerating rat liver has already been recorded[4,6-9,21,22].

The results shown in Table I, however, make it clear that the addition of normal liver extract to the systems derived from ascites cells, regenerating rat liver or rabbit bone marrow causes a pronounced diminution both in the phosphorylation of thymidine and in polymerase activity. Such an inhibitory effect is also found with extracts of kidney and with serum but not with extracts of other tissues such as brain.

The fact that the inhibitory action of normal liver extracts is confined to the thymidylate system and is not observed with the kinases for dCMP, dGMP and dAMP focuses attention on the control of the formation of TTP in the regulation of

DNA biosynthesis. This is in agreement with the views of POTTER[23,24] and of CANELLAKIS *et al.*[22].

The fact that the activity of TdR kinase in extracts of normal liver is increased on ammonium sulphate fractionation suggests that the low activity of the enzyme in unfractionated extracts is due in part at least to the presence of interfering factors.

Although tissue extracts which reduce phosphorylation of TdR also inhibit the polymerase (Table I, IV, V) the inhibition of the two systems is not always in parallel (Table IV). This suggests that different factors are involved.

While the exact nature of these inhibitory factors is still under investigation, it is already clear that the effect is not due to simple dephosphorylation of the substrates or products[10].

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