Biochem. J. (1968) **108**, 541
Printed in Great Britain

Thymidine Kinase in Rat Liver during Development

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(*Received* 16 *January* 1968)

1. The activity of thymidine kinase in rat liver supernatant decreased with development to a value in the adult that was 1% of that in the 17-day foetus. 2. The foetal enzyme was more stable than the adult to gel filtration on Sephadex G-25 at 0°. 3. The greater stability of the foetal enzyme to incubation at 45° was attributable to the presence of higher concentrations of nucleotides in foetal liver supernatant. 4. The K_m values for foetal and adult enzymes were approx. 2·5 μM- and 2·1 μM-thymidine respectively. 5. The foetal enzyme was more sensitive to inhibition by thymidine triphosphate. 6. The decline in enzyme activity during the neonatal period was correlated with a shift in the enzyme properties from the foetal to the adult type, and may reflect the decrease in the proportion of haemopoietic tissue in the liver.

The foetal rat liver is a site of rapid growth (see, e.g., Auerbach & Waisman, 1959) and DNA synthesis (Geschwind & Li, 1949), but these activities decrease towards birth and in the postnatal period. At the same time the predominant proportion of haemopoietic and vascular tissue in foetal liver decreases and the structure approaches that characteristic of adult liver (Oliver, Blumer & Witham, 1963).

Increased thymidine kinase activity is found in many proliferating animal tissues such as regenerating liver (e.g. Bollum & Potter, 1959) and tumours (e.g. Weber & Lea, 1966), and in foetal rat liver, where Bresnick, Thompson, Morris & Liebelt (1964) have also reported that the enzyme is more sensitive to inhibition by thymidine triphosphate than in the adult. The increase in thymidine kinase activity induced in cells by infection with DNA viruses is also associated with changes in the biochemical properties (see, e.g., Klemperer, Haynes, Shedden & Watson, 1967), which reflect a molecular difference between the host and virus-specific enzymes. This approach has been applied to thymidine kinase in foetal and adult liver supernatant, and the biochemical differences are used to follow the change in the enzyme during development.

MATERIALS AND METHODS

Animals. Wistar-derived albino rats were maintained under conventional conditions. Males (3–6 months old, 250–350g.) served as the source of adult liver. To obtain foetuses of known age, six female rats were placed in a cage with a buck for a period of 40hr. and the foetal age was taken from the middle of this period. Thus the 17-day foetuses were those obtained 16 days after removal of the buck.

Labelled thymidine. [2-^{14}C]Thymidine was obtained from The Radiochemical Centre, Amersham, Bucks. The specific radioactivity (determined by counting samples dried on 2·5cm. × 2·5cm. areas of DEAE-cellulose paper under the same conditions, as described below) was 78×10^6 counts/min./μmole.

Determination of thymidine kinase activity. The livers were dispersed at 0° in 5 vol. of 0·25M-sucrose–0·02M-tris–HCl buffer (pH 7·4)–4mM-$MgCl_2$ with ten strokes at 2500 rev./min. in a glass homogenizer fitted with a Teflon pestle (0·1mm. clearance). The homogenate was centrifuged at 0° for 60min. at 100000*g*. After removal of the lipid layer, the upper half of the supernatant was withdrawn and used as the enzyme preparation (foetal preparation approx. 10mg. of protein/ml., neonatal preparation 10–15mg. of protein/ml., adult preparation 20mg. of protein/ml.). Experiments with the foetal enzyme were usually performed with preparations from 19-day or 21-day foetuses. These showed essentially similar characteristics.

The optimum conditions for both foetal and adult liver enzymes were similar to those described by Bresnick & Thompson (1965). With both enzyme preparations the reaction showed an optimum at pH 8·0 (rates at pH 7·5 and 8·5 were approx. 70% of maximum with the adult preparation and approx. 85% of maximum with the foetal preparation). The reaction mixtures (total vol. 0·25ml.) contained liver supernatant from foetal (0·005–0·015ml., 0·05–0·15mg. of protein), newborn (0·03ml., approx. 0·3mg. of protein) or adult animals (0·03ml., approx. 0·6mg. of protein), together with (final concns.) 10 μM-[2-^{14}C]thymidine, 5mM-ATP, 2·5mM-$MgCl_2$ and 0·05M-tris–HCl buffer, pH 8·0. Under these conditions the rate of the reaction was constant for 10–15min. and was proportional to enzyme concentration.

The tubes were incubated for 5 or 10min. at 37° and then heated for 2min. at 100° and cooled quickly in ice. Denatured protein was removed by centrifugation for 10min. at 2000*g* and 0·05ml. samples of the supernatants were applied to 2·5cm.-wide strips of DEAE-cellulose paper

(Sheinin, 1966). Descending chromatography for 2hr. in 1·0mM-ammonium formate removed labelled thymidine and left the phosphorylated derivatives in a 5cm. region that included the origin. Areas (2·5cm. × 2·5cm.) from this region were placed in vials containing 12ml. of toluene and scintillator [4g. of 2,5-diphenyloxazole and 0·05g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.] for measurement of the radioactivity in a liquid-scintillation counter (efficiency approx. 75%, background 35 counts/min.). The radioactivity was corrected for the blank value found for unincubated reaction mixtures. Chromatography of the ^{14}C-labelled reaction product on DEAE-cellulose paper (Ives, Morse & Potter, 1963) showed that it consisted of at least 90% of thymidylic acid together with smaller amounts of thymidine diphosphate and triphosphate. The conversion of labelled thymidine into phosphorylated derivatives was therefore a measure of thymidine kinase activity.

Pretreatments of enzyme preparations. Liver supernatants were subjected to gel filtration at 0° by passing the sample (0·5ml.) through a 20cm. × 1cm. column of Sephadex G-25 that had been previously equilibrated with 0·05M-tris–HCl buffer, pH 8·0, containing in some cases the substance (e.g. 1mM-ATP) to be tested as a stabilizer of enzyme activity. All of the protein was eluted from the column in the first 1ml. immediately following the void volume. This material was taken for determination of enzyme activity.

For heat-inactivation experiments the supernatants were mixed with 0·05vol. of 1·0M-tris–HCl buffer, pH 8·0, and heated in a water bath at 45°. Samples were taken at intervals and assayed immediately for enzyme activity.

To prepare boiled extracts the liver supernatants were heated for 2min. at 100°, cooled to 0° and centrifuged for 10min. at 20000*g* to remove denatured protein.

Determination of protein. Protein in the liver supernatants was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Thymidine kinase activity during development. Table 1 shows that high activities of thymidine kinase were found in supernatants from livers of 17-day foetuses. Thereafter the activity decreased rapidly towards term. During neonatal development the decrease was more gradual and the final adult enzyme activity was about 1% of that in the 17-day foetus. The amount of reaction product formed by mixtures of foetal and adult preparations was the sum of the amounts of reaction product formed by the component enzymes separately (see Table 2), and in other experiments the activity of neither enzyme was significantly affected by the addition of a boiled preparation of the other. Therefore the marked differences in enzyme activity at different stages of development did not appear to be due to the presence of activating or inhibitory factors.

Stability of foetal and adult enzymes. Table 2 shows that the foetal enzyme was more stable than the adult enzyme to gel filtration. After passage over a column of Sephadex G-25 the adult enzyme lost over 90% of its activity whereas the foetal enzyme lost about 75% of its activity. Of a number of substances tested (including thymidine, thymidine triphosphate, β-mercaptoethanol and glycerol) only ATP stabilized the enzymes under these conditions. Thus in the presence of 1mM-ATP 20–25% of the adult enzyme activity and 80–90% of the foetal enzyme activity were recovered after gel filtration. When mixtures of the foetal and adult enzymes were passed over Sephadex columns the enzyme activity recovered was the sum of the activities of the two preparations treated separately. Similar results were obtained when the foetal preparation was diluted to give about the same enzyme activity as the adult liver supernatant (Table 2, Expt. B). The greater loss of activity in the adult preparation was therefore not due to the presence of inactivating agents, but probably reflected a relative instability of the adult enzyme.

Because of the low recovery of adult enzyme activity after gel filtration other comparisons between the two enzyme preparations were made with the untreated liver supernatants. It is possible that under these circumstances some of the apparent differences between foetal and adult preparations actually reflected differences in the amounts of diffusible molecules. This is illustrated by the

Table 1. *Decrease in thymidine kinase during development*

Enzyme activity (expressed as the specific activity, μμmoles of thymidine phosphorylated/min./mg. of protein) was measured in the presence of 10μM-[2-^{14}C]thymidine, 5mM-ATP, 2·5mM-$MgCl_2$ and 0·05M-tris–HCl buffer, pH 8·0. The reaction mixtures contained (in 0·25ml. total vol.) liver supernatant from foetal (0·05–0·15mg. of protein), neonatal (approx. 0·3mg. of protein) or adult animals (approx. 0·6mg. of protein). After an incubation at 37° for 5min. (foetal preparation) or 10min. (newborn or adult preparation) the incorporation of radioactivity into thymidine phosphates was measured as described in the Materials and Methods section. Each value represents the enzyme activity in a supernatant prepared from the combined livers of all foetuses in one litter, or from the combined livers of three newborn animals taken at random from one litter, or from individual livers of adult males (250–350g.).

Animals from which enzyme preparation obtained	Thymidine kinase activity (μμmoles/min./mg. of protein)		
Foetus (17-day)	2180	2200	2500
Foetus (19-day)	1166	1310	1630
Foetus (21-day)	292	362	624
Newborn (3-day)	372	384	388
Newborn (7-day)	226	226	232
Newborn (14-day)	73	81	114
Newborn (35-day)	26	30	35
Adult	12	14	20

Table 2. *Effect of gel filtration on enzyme activity*

In Expt. A liver supernatants from adult (20·9mg. of protein/ml.) and 21-day foetus (10·2mg. of protein/ml.) were tested separately or as a mixture (2vol. of adult preparation + 1vol. of foetal preparation + 1vol.of 0·05M-tris–HCl buffer, pH8·0). Samples (0·5ml.) of supernatant or mixture were applied to columns of Sephadex G-25 equilibrated with 0·05M-tris–HCl buffer, pH8·0, containing 1mM-ATP where indicated. The protein was recovered in 1ml. of eluate. Reaction mixtures similar to those described in Table 1 were incubated for 5min. at 37° and contained (in 0·25ml.) 0·03ml. of adult supernatant, or 0·015ml. of foetal supernatant, or 0·03ml. of mixed supernatants (equivalent to 0·015ml. of adult + 0·0075ml. of foetal). After gel filtration these volumes of supernatant were doubled to compensate for the dilution (from 0·5ml. to 1·0ml.). Enzyme activity is expressed as $\mu\mu$moles of thymidine phosphorylated/5min./0·25ml. reaction mixture. The expected activity of the mixed supernatants is half the sum of the activities of the adult and foetal preparations measured separately. Expt. B was similar except that conditions were such that approximately similar enzyme activities were measured with the untreated preparations from adult (22·0mg. of protein/ml.) and 21-day foetus (diluted with homogenizing medium to 2·7mg. of protein/ml.).

Treatment of liver supernatant	Thymidine kinase activity ($\mu\mu$moles/5min.) of liver supernatant		
	Adult	Foetal	Adult + foetal
Expt. A			
No treatment	42	258	159
Sephadex	3	64	35
Sephadex (1mM-ATP)	9	215	108
Expt. B			
No treatment	54	70	63
Sephadex	4	21	11
Sephadex (1mM-ATP)	12	65	38

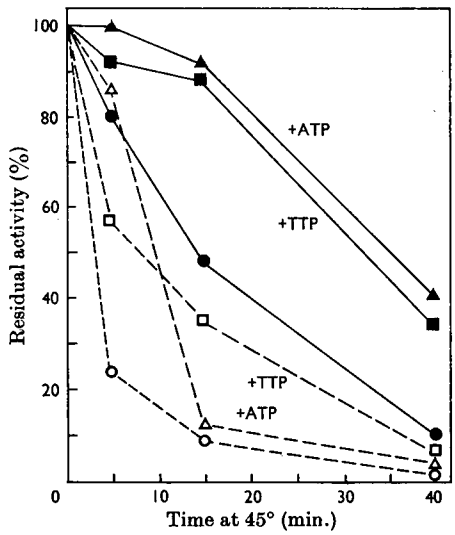

Fig. 1. Enzyme inactivation by incubation at 45°. Supernatants from adult liver (○, 20·4mg. of protein/ml.) and from 19-day foetal liver (●, 11·2mg. of protein/ml.) were heated at 45° in the presence of 0·05M-tris–HCl buffer, pH8·0, and, where indicated, of 1·0mM-ATP (△, adult; ▲, foetal) or of 5μM-thymidine triphosphate (TTP) (□, adult; ■, foetal). Samples (0·03ml. of adult, 0·015ml. of foetal) were taken at intervals for determination of thymidine kinase activity as described in Table 1. The activities ($\mu\mu$moles of thymidine phosphorylated/0·25ml. reaction mixture) are expressed as percentages of the activity before heating. These control values were 100$\mu\mu$moles/10min. and 1250$\mu\mu$moles/5min. for adult and foetal enzyme respectively.

apparent resistance of the foetal enzyme to heating.

Fig. 1 shows that the foetal enzyme was more stable than the adult enzyme to heating at 45°, but in the presence of ATP (1mM) or of thymidine triphosphate (5μM) both enzymes were relatively stable for short periods. The greater apparent stability of the foetal enzyme may therefore actually have depended on contamination with larger amounts of these nucleotides. In agreement with this, other experiments showed that after Sephadex treatment the foetal enzyme was very rapidly inactivated at 45° unless stabilized by the presence of ATP or thymidine triphosphate, and that when the foetal enzyme was diluted in the adult preparation the rate of inactivation became similar to that observed with the adult enzyme.

Effects of substrate concentration and of thymidine triphosphate. Measurements of the change in reaction velocity with substrate concentration gave similar kinetics for both enzymes (Fig. 2) and gave a K_m value for the foetal enzyme (2·5μM-thymidine) that was only slightly higher than that of the adult enzyme (2·1μM). These values may not be significantly different.

On the other hand there were marked differences in sensitivity to inhibition by thymidine triphosphate (Fig. 3). Under the assay conditions employed here, 30μM-thymidine triphosphate inhibited the foetal enzyme by 70%, whereas the adult enzyme was only 20% inhibited. Other experiments showed that the percentage inhibition by thymidine triphosphate was not affected by the amount of enzyme or by the time of incubation, and (see Fig. 3 legend) in the presence of thymidine triphosphate the enzyme activity of a mixture of foetal and adult preparations was the sum of their activities when tested separately. Further, the inhibition of neither adult nor foetal preparation was affected by the presence of a boiled extract of the other (Table 3). The different sensitivities to thymidine triphosphate therefore did not reflect the actions of other components (e.g. of diffusible

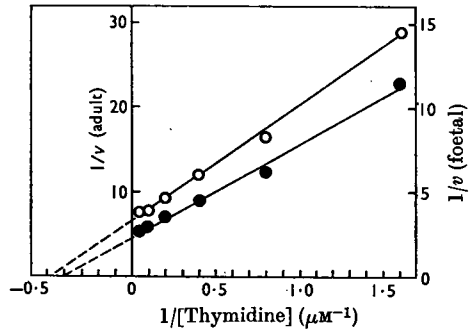

Fig. 2. Increase in reaction velocity with substrate concentration: double-reciprocal plots of velocity, v (mμmoles of thymidylate synthesized/0·25 ml.), against substrate concentration. The incubation conditions were as described in Table 1 except that the [^{14}C]thymidine concentration varied between 0·625 μM and 20·0 μM as indicated. The values presented are: ○, adult (0·6 mg. of protein/0·25 ml., incubated for 10 min.), intercept = −0·47, K_m = 2·1 μM (two other experiments gave values of 1·9 and 2·0 μM); ●, 19-day foetus (0·07 mg. of protein/0·25 ml., incubated for 5 min.), intercept = −0·4, K_m = 2·5 μM (two other experiments gave values of 2·4 and 2·5 μM).

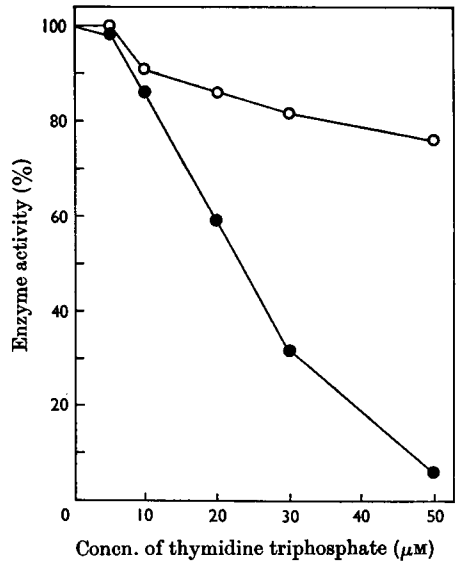

Fig. 3. Inhibition by thymidine triphosphate. The reaction mixtures were as described in Table 1 except that thymidine triphosphate was present at the final concentration shown. The tubes contained (in 0·25 ml.) enzyme from adult (0·54 mg.) or 21-day foetus (0·09 mg.), and both sets were incubated for 10 min. Enzyme activities (○, adult; ●, 21-day foetus) are expressed as percentages of the activity without thymidine triphosphate. Thymidine phosphorylated/0·25 ml./10 min. in the absence of thymidine triphosphate by adult and foetal enzymes respectively was 82 mμmoles and 346 mμmoles. In the presence of 30 μM-thymidine triphosphate the corresponding values were 67 mμmoles and 109 mμmoles. With both enzymes together in the presence of 30 μM-thymidine triphosphate the value was 167 mμmoles (compare theoretical 67 + 109 = 176 mμmoles).

molecules or of enzymes that destroy the inhibitor), but reflected the properties of the enzymes themselves.

Changes in enzyme characteristics during the neonatal period. Table 4 shows that the characteristics of the enzyme changed from foetal to adult during the neonatal period. With increasing age the sensitivity to inhibition by thymidine triphosphate decreased, and less activity was recovered after gel filtration in the presence of 1 mM-ATP. These results suggest a gradual replacement of the foetal by the adult enzyme.

DISCUSSION

Bresnick *et al.* (1964) have already briefly reported that thymidine kinase activity is higher in foetal liver than in the adult, and that the foetal enzyme is more sensitive to inhibition by thymidine triphosphate. The main new finding in the present paper is that the foetal enzyme is more stable to gel filtration. In addition, the decrease in enzyme activity during foetal and neonatal development is presented in more detail and correlated with a transition in the properties of the enzyme to those characteristic of the adult type.

The use of the untreated liver supernatants for comparisons of the adult and foetal enzymes was necessitated by the low recovery of adult enzyme after gel filtration, but this meant that some apparent differences between the two enzyme preparations could actually have reflected different concentrations of diffusible molecules such as sub-

Table 3. *Inhibition by thymidine triphosphate in the presence of boiled extracts*

Thymidine kinase activity was measured in liver supernatant (0·03 ml.) from adult (20·4 mg. of protein/ml.) and 21-day foetal animals (4·6 mg. of protein/ml.). The reaction mixtures (0·25 ml.) were incubated for 5 min., and were as described in Table 1 except that a boiled extract of liver supernatant (0·03 ml.) and 30 μM-thymidine triphosphate (TTP) were added as indicated.

Liver supernatant (0·03 ml.)	Boiled extract (0·03 ml.)	Thymidine kinase activity (μμmoles/5 min.) Standard conditions	30 μM-TTP present
Adult	—	48	39
Adult	Foetal	49	39
Foetal	—	347	120
Foetal	Adult	345	113

Table 4. *Changes in properties of thymidine kinase during development*

Samples of the preparations (two from each stage of development) shown in Table 1 were tested as described in Table 1, either in the presence of 30 μM-thymidine triphosphate (TTP), or after mixing in equal proportions and gel filtration on Sephadex G-25 (in 1mM-ATP–0·05M-tris–HCl buffer, pH 8·0). Enzyme activities are expressed as percentages of the activity in the absence of thymidine triphosphate or before gel filtration.

Animals	Thymidine kinase activity (% of control)		
	In 30 μM-TTP		After gel filtration
Foetus (21-day)	32	35	88
Newborn (3-day)	44	44	85
Newborn (14-day)	48	53	68
Newborn (35-day)	64	70	46
Adult	74	81	23

strates and allosteric effectors (see, e.g., Consigli & Ginsberg, 1964). Further, the relatively low activity of the adult enzyme required the use of about five-fold more adult than foetal liver supernatant for determinations of enzyme activity. For this reason incubations with the adult enzyme would receive a relatively larger proportion of the effector molecules originally present in the supernatant, as well as containing more total protein, and might therefore have contained larger amounts of protein or non-protein activators and inhibitors.

The influence of these extraneous factors on the properties of each enzyme may be exposed by a comparison with the properties of the two enzymes mixed together. For example, the relative thermal stability of the foetal enzyme was lost when it was diluted with the adult preparation, and other evidence suggested that this stability depended on the presence of higher concentrations of nucleotides. On the other hand, when the two enzymes were mixed and either passed over a Sephadex column (which would in any case eliminate differences due to diffusible substances) or tested in the presence of thymidine triphosphate, the activity was the sum of their individual activities under the same conditions. Therefore the relative stability to gel filtration and the greater thymidine triphosphate inhibition of the foetal enzyme were real characteristics and indicate that the thymidine kinase activities in foetal and adult preparations were due to different enzymes.

These characteristics suggest that the activity of the enzyme in foetal liver could be regulated mainly by feedback inhibition by thymidine triphosphate. In contrast, the instability of the enzyme in adult liver, which has been observed also *in vivo* (Bresnick, Williams & Mosse, 1967), would allow the total amount of enzyme to be controlled by the rate of synthesis of the corresponding messenger RNA, which itself is known to have a short half-life (Pitot, Peraino, Lamar & Kennan, 1965; Bresnick *et al.* 1967).

The decrease in the enzyme activity during development coincided approximately with the disappearance of haemopoietic and vascular tissue, which has been shown to fall from 60% in 17-day foetal liver to about 10% at 7 days after birth (Oliver *et al.* 1963). The transition in enzyme properties during the neonatal period may therefore reflect the change in liver histology, just as, for example, the developmental change in kidney lactate dehydrogenase isoenzymes has been shown to reflect the changing proportions of the different renal tissue components (Smith & Kissane, 1963). In agreement with this, preliminary experiments show that high thymidine kinase activities occur in the red bone marrow from the femur of 6-week-old rabbit, and that this enzyme behaves like the foetal liver enzyme on gel filtration and in the presence of thymidine triphosphate. Assuming that the activity of thymidine kinase in liver is relatively high in the mitotically active haemopoietic cells (foetal type) compared with hepatic cells (adult type), then adult properties would only be demonstrable during the postnatal period when the haemopoietic cells contribute a less overwhelming proportion of the total enzyme activity.

The high thymidine kinase activity in foetal liver, as in other rapidly growing tissues, again suggests some special function in relation to DNA synthesis. The enzyme catalyses the first step of a 'salvage' pathway leading to thymidine triphosphate and so contributes one of the precursors for DNA synthesis, but it may have a further significance beyond this. Moore & Hurlbert (1966) found that the reduction of the ribonucleoside diphosphates to deoxyribonucleoside diphosphates by a mammalian enzyme required activation by low concentrations of ATP and thymidine triphosphate. Thus the availability of thymidine triphosphate via an independent pathway may be a way in which the synthesis of all four deoxyribonucleotides and hence of DNA can be controlled. Thymidine kinase would then be the first enzyme of a pathway for synthesizing a molecule with a regulatory function.

This work was supported in part by a Research Grant from the Medical Research Council. We thank Mr D. Hickman-Smith for advice on breeding and Mr A. Smith for the care of the animals. We are also grateful to Pfizer Ltd. for financial assistance.

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