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THYMIDYLATE KINASE ACTIVITY IN MALE RAT ANTERIOR PITUITARY DURING GROWTH

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SUMMARY

1. ATP:thymidine monophosphate phosphotransferase (EC 2.7.4.9) is present in the anterior pituitary post-mitochondrial supernatant of the male albino rat. It is the rate-limiting enzyme for dTMP phosphorylation into dTTP, as shown by electrophoretic analysis of the incubation medium.

2. This enzyme requires ATP, Mg^{2+} and 3-phosphoglycerate to exhibit full activity. It is a very labile enzyme, for homogenization must be performed in the presence of dTMP so as to protect it against loss of activity; preincubation at 37 °C in the absence of substrate inactivates the enzyme in a few minutes. β-Mercaptoethanol in the homogenization medium is not necessary.

3. From the substrate saturation curve, we get an apparent K_m value of $1.1 \cdot 10^{-5}$ M; this value is the same for immature and adult animals.

4. The dTMP kinase activity per gland increases in a sigmoidal fashion, with a sudden rise between 20 and 40 days. It follows that the specific activity curve, after a trough between 10 and 20 days, presents a maximum at 30–35 days and decreases then regularly during rat sexual maturation.

5. NaF, a potent phosphatase inhibitor, also partly inhibits the dTMP kinase activity.

6. The anterior pituitary specific activity is several times higher than that of brain or liver. Liver dTMP kinase inhibitor is also effective on pituitary enzyme.

7. We do not observe any relationship between dTMP kinase activity and DNA biosynthesis in the rat anterior pituitary during growth; we cannot conclude about participation of this enzyme in the regulation of DNA biosynthesis.

INTRODUCTION

The participation of dTMP kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9) in the regulation of DNA biosynthesis has been investigated in several tissues for which DNA synthesis is particularly high: regenerating liver[1],

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rat liver after recovery from protein malnutrition[2], several kinds of tumor cells[3,4]. This enzyme is known to have low activity in tissues which are not highly proliferating (for a more general review see ref. 5).

We have recently shown that development of male rat adenohypophysis goes through three stages and that during the second step—roughly between 25 and 55 days—the ratios of protein, RNA and DNA vary tremendously[6,7]. We have also established that tritiated thymidine is incorporated into DNA during incubation of rat anterior pituitaries and that the amount incorporated decreases during growth from immature animals to adult ones[8]. Others[9–11] have shown further that the labeling index of pituitary decreases continuously during growth.

Following incubation of rat adenohypophysis in the presence of tritiated thymidine, chromatographic and electrophoretic analysis of the acid-soluble fraction reveals that the precursor is partly transformed into dTTP[8]; it follows that one can expect two important enzymatic activities, *i.e.* dTMP and dTDP kinases, for [^{3}H]dTMP and [^{3}H]dTDP are present only in trace amounts.

All these results prompted us to investigate the dTMP kinase activity in the male rat anterior pituitary, to determine its fluctuations during animal growth and try to correlate these fluctuations with DNA increase. The other purpose is to study further the effect of castration and testosterone therapy upon this enzyme, for the ratio of dTTP relative to the other thymidylic nucleotides is modified by gonadectomy[8].

Having in mind some preliminary results[12], we have been brought to investigate the influence of several factors (preincubation, phosphatase inhibitor . . .) on dTMP kinase activity and to compare the pituitary specific activity with that of brain, hypothalamus and liver.

MATERIALS AND METHODS

Animals

Male albino rats of the Wistar strain were purchased from élevage Janvier (53, Le Genest) 2 days after birth, with one mother for 10 pups. When they were 23 days old, mothers were discarded and rats were housed in cages of 5 animals; they were maintained on U.A.R. No. 103 diet and water *ad libitum*, with light from 7 a.m. to 7 p.m.

Chemicals

Thymine, thymidine, dTMP, dTDP and dTTP were purchased from Sigma Chemical Co.; ATP and 3-phosphoglycerate came from Boehringer.

(*Me*-^{3}H]dTMP (12 Ci/mmole) was from the Radiochemical Centre, Amersham.

Preparation of post-mitochondrial supernatant

Rats were killed with a guillotine (Harvard Apparatus Corp.). The anterior pituitaries (3–5, according to rat age) were removed and immediately homogenized at 0 °C into a micro tissue grinder (0.5-ml capacity) containing 0.150 ml of the following extraction medium: 25 mM phosphate buffer (pH 7.4), 0.4 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.002 mM dTMP. This buffer is derived from Kielley's medium[3]. Non-radioactive dTMP is added into the extraction medium in order to avoid rapid in-

activation of the enzyme. In some experiments (see Results), NaF (25 mM) was added to the solution as an inhibitor of the various phosphatases.

The homogenate was spun down at 800 × *g* for 10 min; the supernatant was centrifuged at 20 000 × *g* for 10 min. The 800 × *g* pellet is used for DNA determinations by ultraviolet absorption according to the method previously described[6], whereas the new supernatant (post-mitochondrial fraction) is used as the enzyme source. The protein content is determined by the method of Lowry *et al.*[13].

dTMP kinase activity

0.080 ml of the enzyme fraction was added to 0.020 ml of Tris–HCl buffer (pH 7.4) containing substrate and phosphate donor and incubations were performed at 37 °C, in the following incubation medium (final concentrations): 20 mM phosphate buffer (pH 7.4), 40 mM Tris–HCl buffer (pH 7.4), 16 mM $MgCl_2$, 8 mM ATP, 32 mM 3-phosphoglycerate, 0.32 mM β-mercaptoethanol, 0.2 mM [*Me*-^{3}H]dTMP (0.48 μCi; specific activity 24 mCi/mmole). For some experiments, the medium is 20 mM NaF.

The incubation is stopped by pipetting an aliquot (usually 0.025 ml) and transferring it into a boiling-water bath 1 min. The protein fraction is discarded by centrifugation at 3000 × *g* for 10 min at 2 °C; the supernatant (0.005 ml) is immediately analyzed by high-voltage electrophoresis (Pherograph), using Whatman 3MM

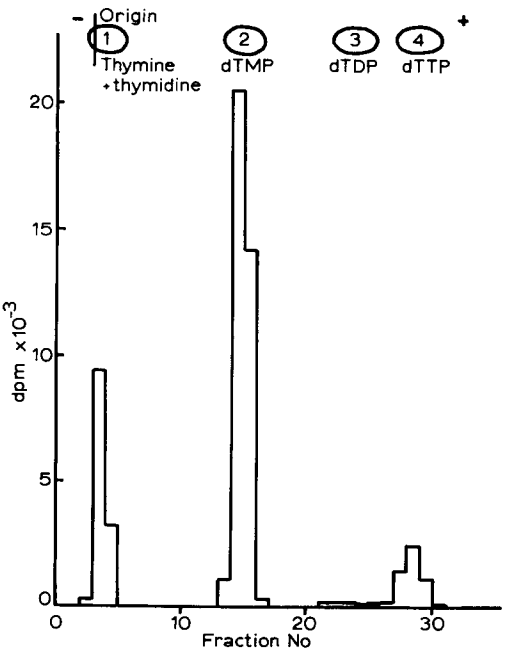

Fig. 1. Five pituitaries are homogenized in NaF-free extraction medium, and 30 000 × *g* supernatant is used as the enzyme source. Incubation is performed for 30 min at 37 °C in 0.1 ml solution containing 2 μmoles KH_2PO_4, 4.0 μmoles Tris, 0.032 μmole mercaptoethanol, 1.6 μmoles $MgCl_2$, 0.8 μmole ATP, 3.2 μmoles 3-phosphoglycerate, 0.005 μmole [^{3}H]dTMP (0.5 μCi). Incubation is stopped by boiling for 2 min. After centrifugation, 0.005 ml supernatant is analyzed by high-voltage electrophoresis in 0.05 M citrate buffer (pH 4.1) at 47 V/cm during 35 min, on Whatman 3MM paper. Countings are performed after elution with 0.01 M HCl.

paper and 0.05 M citrate buffer (pH 4.1) at 47 V/cm for 35 min[8]. The areas corresponding to thymine, thymidine and the various thymidylic nucleotides (as shown with markers running in parallel) are eluted with 0.01 M HCl by capillarity and the droplets are collected directly in the scintillation vials. 10 ml Instagel (Packard) is added to each vial and counting is performed with a SL 30 Intertechnique counting apparatus. Yields are obtained with the use of an external standard.

The enzymatic activity is expressed in nmoles of dTMP phosphorylated (sum of radioactive dTDP *plus* dTTP) per min.

RESULTS

Electrophoretic analysis of the post-incubation supernatant

Fig. 1 shows the elution profile corresponding to the analysis of a post-incubation supernatant. dTDP is present only in a trace amount, which confirms that phosphorylation of dTDP into dTTP proceeds very rapidly and that phosphorylation of dTMP is the rate-limiting step in our assay. Thymidine is present in a rather high amount; there are two reasons for this: first, the commercial tritiated dTMP contains roughly 5% thymidine as an impurity and it has not been further purified; second, the phosphatase activity is not negligible. One can block this last activity completely by incubating in the presence of NaF, but we shall see later that NaF also partly inhibits the dTMP kinase. It follows that in the first group of experiments, we did not use NaF, but we used this inhibitor in the second series and we shall compare the results obtained.

Kinetics and influence of enzyme concentration

Fig. 2 shows that the reaction proceeds linearly for at least 30 min, either with pituitaries of either 34- or 75-day-old rats. For all the subsequent experiments, we routinely incubated for 20 min. In Fig. 3, one sees that the enzymatic activity is

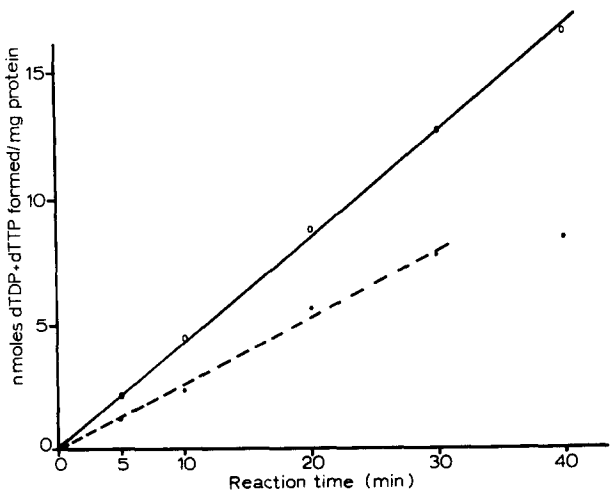

Fig. 2. Kinetics of dTMP kinase activity. Incubation medium is the same as in Fig. 1, but in the presence of 0.020 μmole [^{3}H]dTMP (0.5 μCi). Rats are 34 days old (○—○) or 75 days old (●---●).

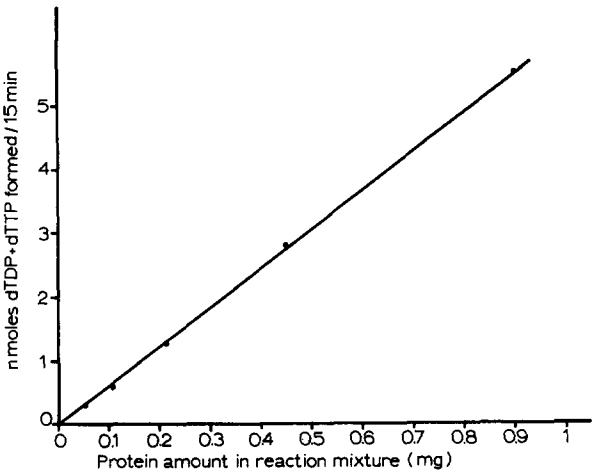

Fig. 3. Influence of enzyme concentration on dTMP kinase activity. Incubation conditions same as in Fig. 2. Rats 34 days old. In abscissa, amount of post-mitochondrial protein per 0.1 ml incubation medium.

proportional to enzyme concentration, up to 9.1 mg/ml postmitochondrial protein. We routinely used 0.2–0.6 mg protein per 0.1 ml incubation medium for the subsequent experiments.

Determination of apparent K_m

We have performed several incubations with increasing amounts of substrate so as to verify first that we were routinely using saturating concentrations, second that the plateau is attained with the same substrate concentrations for immature

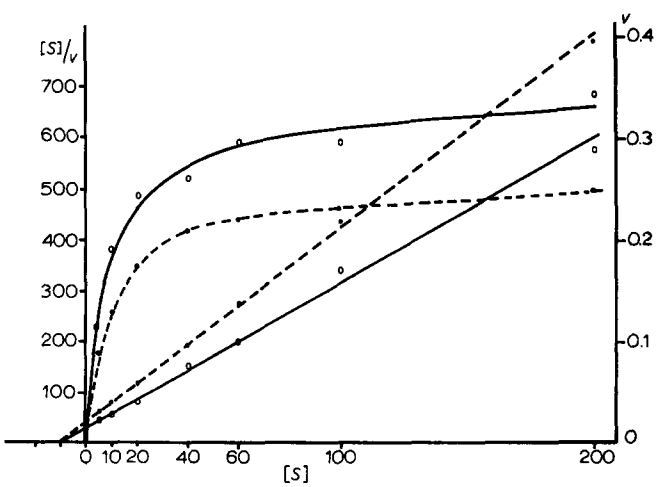

Fig. 4. Influence of substrate concentration on dTMP kinase activity. Incubation conditions same as in Fig. 2. Substrate concentrations are in μM and initial velocity (v) is nmoles dTMP phosphorylated per min and per mg post-mitochondrial protein. K_m is estimated using Woolf's representation $[S]/v = f[S]$.

rats as for adult ones, and third to estimate an apparent K_m for the dTMP kinase.

We see (Fig. 4) that this enzymatic reaction follows Michaelis–Menten kinetics; specific activity is not identical for young rats and adult ones, but the concentration of substrate routinely used is located on the horizontal part of the curve. The K_m obtained, $1.1 \cdot 10^{-5}$ M, is the same for both kinds of animals. Furthermore, we do not record any substrate inhibition as had been mentioned for purified enzyme[3].

Effect of omission of various components in extraction and incubation media

We have reported in Fig. 5 the specific activities obtained after incubation in various media, as a percentage of the activity given by complete medium. One can

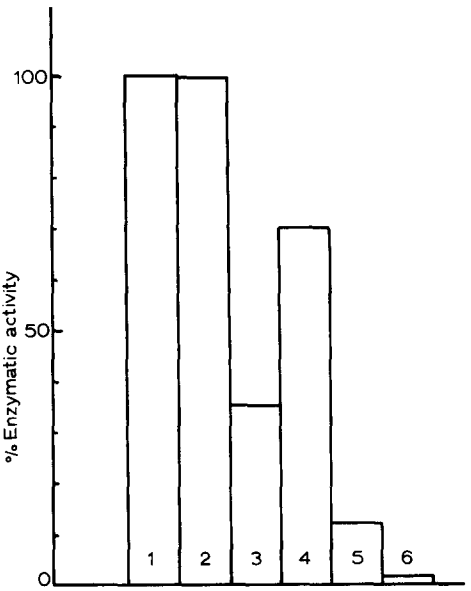

Fig. 5. Effect of various components on dTMP kinase activity. (1) Complete medium as defined in Methods, without NaF; homogenization medium without mercaptoethanol (2) or without dTMP (3); incubation medium without 3-phosphogylcerate (4), ATP (5) or Mg^{2+} (6).

see that the enzyme requires ATP, Mg^{2+} and 3-phosphoglycerate to prevent ATP hydrolysis. On the other hand, β-mercaptoethanol is not necessary, but as we used it in our first experiments, we routinely used it in the succeeding ones.

dTMP kinase is a very unstable enzyme, for it requires a small amount of substrate in the extraction medium to get full activity. Moreover, if we preincubate the enzyme solution at 37 °C only in the presence of the limited amount of dTMP included in the extraction medium, the activity falls off to 20% activity after 3 min preincubation and becomes quite undetectable after 15 min preincubation.

Variation of activity during rat growth

We have reported in Fig. 6 the results obtained in terms of rat age, all the animals of this series being bred in our laboratory. The dTMP kinase activity per gland

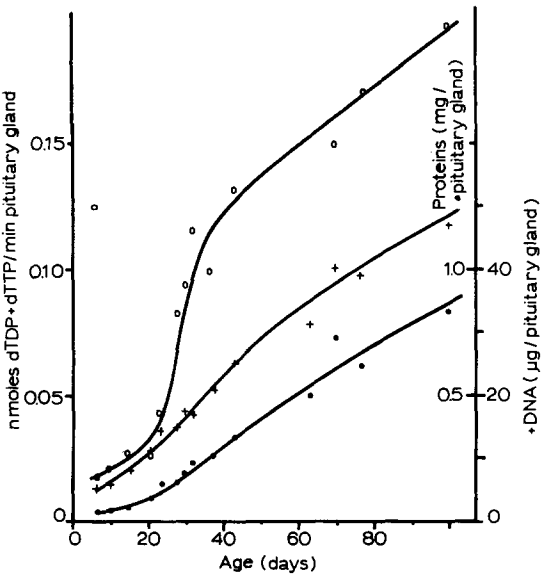

Fig. 6. Variation of dTMP kinase activity during rat growth. Experimental conditions according to Methods; incubation in NaF-free medium. μg DNA per pituitary (+—+), mg post-mitochondrial protein per gland (●—●), nmoles dTDP + dTTP formed per min per pituitary (○—○). Each point represents one experiment, but three electrophoretic analyses are performed after 10, 20 and 30 min incubation so as to check linearity of the reaction and get more accurate determination of initial velocity.

increases in a rather sigmoid fashion, with a sudden rise between 20 and 40 days. In this figure are also expressed the DNA and post-mitochondrial protein amounts per gland; they do not show the same pattern as the enzyme curve, for DNA and proteins increase more regularly.

These results are best expressed by derivative curves which show the daily increase of each parameter (Fig. 7). The daily increase in DNA remains constant until roughly 40 days and then diminishes regularly; this result is in agreement with the decrease of labeling index during rat growth[9,10]. The daily post-mitochondrial protein increase reaches a maximum at approx. 30 days and then slows down. On the other hand, the daily increase in dTMP kinase is represented by a bell-shaped curve with a well defined maximum between 25 and 30 days.

The specific activity is reported in Fig. 8a; it decreases between 10 and 20 days, goes through a maximum for 30–35 days and slows down regularly, the adult value being approximately half the maximum value. We have also reported in this figure the results we got with another serie of animals, pituitaries being incubated in the presence of NaF. Though the rats did not come from an homologous series (only some of them were bred in our laboratory), one gets the same shaped curve, with a trough as in the first series.

In order to get an idea of the variation of mean enzymatic activity per cell, we have expressed (Fig. 8b) the activity for μg DNA. A maximum is reached a few days after weaning, followed by a small decrease; the per cell activity remains constant after the animal becomes mature.

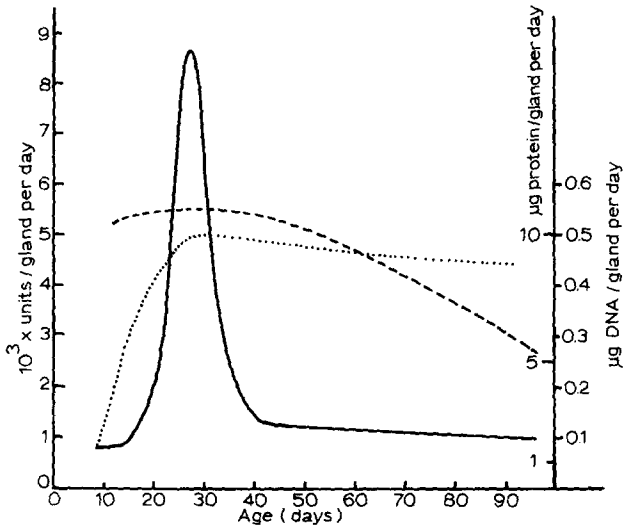

Fig. 7. Daily increase of dTMP kinase activity during rat growth. These curves have been calculated from those reported in Fig. 6, using 3-day intervals. It represents a per day and per gland increase. One enzymatic unit is defined as the number of nmoles of dTMP phosphorylated per min. Enzymatic activity (———), proteins (. . . .), DNA (— — —).

Influence of NaF on dTMP kinase activity

Though we have seen in the preceding figures that enzymatic activity tested with or without NaF is represented by the same shaped curves, one can mention that the values are not exactly the same. We decided to test the influence of NaF in the incubation medium. Three experiments have been performed; in the first one, the pituitaries of different rats have been used to compare activities; in the two last

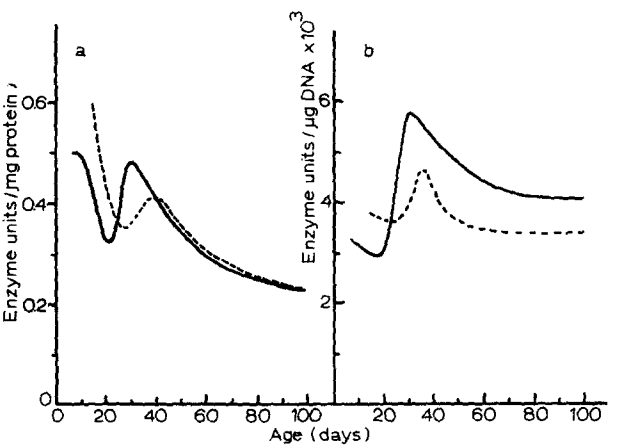

Fig. 8. a. Specific activity of dTMP kinase during rat growth. Experimental conditions, see Methods. Curves calculated from curves of Fig. 6 in the case of NaF-free incubation medium (———) and from curves not represented in this paper corresponding to incubations in 20 mM NaF-containing medium (— — —). b. Enzymatic activity per µg DNA as a function of rat age. Same conditions as for a.

TABLE I

INFLUENCE OF NaF ON PITUITARY dTMP KINASE ACTIVITY

For the first experiment, 3 pituitaries are homogenized in 0.150 ml of NaF-free extraction medium and 3 other glands in 25 mM NaF-containing medium. For the 2nd and 3rd experiments, respectively, 7 and 5 pituitaries are divided into halves and homogenized in appropriate medium. It follows that incubation solution contains or not 20 mM NaF. One unit is equal to the number of nmoles of dTMP phosphorylated per min. Specific activity is the number of units/mg post-mitochondrial protein.

Experiment No.	*Rat age (days)*	*Specific activity*		*Inhibition (%)*
		−NaF	*+NaF*	
1	35	0.41	0.39	4.8
2	35	0.43	0.34	20.9
3	37	0.54	0.38	29.6

experiments, we divided each pituitary into halves, one half being incubated without NaF, the second one with NaF.

One sees (Table I) that NaF partly inhibits the dTMP kinase activity (mean: 18.3% inhibition).

Comparison of dTMP kinase activity in different rat tissues

As the pituitary specific activity seemed to be rather high, we decided to test this dTMP kinase activity in several tissues. We used liver, known to possess a low level of activity, anterior part of brain and hypothalamus. One can see (Table II) that we obtain the highest specific activity with anterior pituitary; it represents in fact 9–10 times the activity of brain and hypothalamus and 4–10 times that of liver.

As Fiala and Fiala[4] have shown that liver possesses a microsomal inhibitor of dTMP kinase activity, we investigated the effect of this inhibitor on pituitary kinase activity by mixing equal amounts of liver and pituitary post-mitochondrial supernatants. The activity of the mixture is less than half the sum of the individual activities (Table II), showing that the liver inhibitor is also effective with pituitary enzyme.

TABLE II

dTMP KINASE ACTIVITY IN VARIOUS RAT TISSUES

For Expt 1. hypothalamus, parts of liver and anterior brain of one rat are used to test the enzymatic activity. For experiments 2 and 3, 6 pituitaries and one part of liver (approximately same weight as the 6 glands) are homogenized in 0.300 ml of extraction solution. 0.080 ml of pituitary and liver supernatants are incubated individually and 0.040 ml of each solution are mixed prior to incubation. All incubations are performed in the presence of 20 mM NaF. Protein amount is estimated in each solution to permit determination of individual specific activities and theoretical specific activity of mixture.

Experiment No.	*Rat age (days)*	*Specific activity*						*Inhibition (%)*
		Hypothalamus	*Brain*	*Pituitary*	*Liver*	*Mixture*	*Theoretically*	
1	54	0.038	0.042	0.35*	0.028	—	—	—
2	30	—	—	0.321	0.071	0.144	0.196	26.5
3	31	—	—	0.358	0.101	0.180	0.229	21.3

* This value does not come from the same animal as the one used for hypothalamus, brain and liver determinations, but it is a mean value for this rat age.

DISCUSSION

We have shown in this study that dTMP kinase functions in rat anterior pituitary. This enzyme, like other kinases, requires ATP and Mg^{2+}; β-mercaptoethanol is not necessary to protect the enzyme from oxidation. The enzyme is very unstable, and homogenization must be done in the presence of a small amount of substrate so as to protect it against inactivation; furthermore, one should avoid preincubation, the enzyme being added last to start the reaction. Our results agree with Kielley's conclusions[3], except for the thiol compound requirement.

Ives[14] has shown from Novikoff Hepatoma cells that phosphorylation of dTMP into dTTP proceeds through preliminary phosphorylation into dTDP, and that nucleoside diphosphokinase is more than 1000 times as active as thymidylate kinase. Our electrophoretic analysis leads to the same conclusion, since dTDP is always found in trace amounts.

Our determination of the effect of increasing substrate concentration on dTMP kinase activity gives an apparent K_m value of $1.1 \cdot 10^{-5}$ M, for both immature and adult rats. Two values have been recently reported: $5 \cdot 10^{-4}$ M for *Rana pipiens* embryos[15], and $1.9 \cdot 10^{-4}$ M for purified enzyme from mouse ascites hepatoma[3]. Our value is 20–50 times less than others, which represents a much higher affinity of the pituitary enzyme for its substrate. Contrary to Kielley[3], we do not observe any inhibition by excess substrate.

Another point to be mentioned is the inhibitory effect of NaF. This effect of fluoride has already been mentioned, as phosphorylation of dTMP by regenerating rat liver homogenate is seriously impaired by 4 mM KF[1]. As we neither tested various concentrations of inhibitor, nor tried to purify the enzyme, one cannot decide whether one or several kinds of dTMP kinases are present in anterior pituitary.

The modification of dTMP kinase activity during rat growth seems to be very interesting. Pituitary is known to be an organ without cell renewal[16]; during animal growth, the labeling index decreases continuously[9–11]. Our results show that whereas DNA biosynthesis occurs at a daily decreasing rate, dTMP kinase activity shows a sudden rise just after weaning; we have no explanation for this increase in specific activity, the maximum of which is attained at about 35 days. There is no apparent correlation between DNA synthesis and dTMP kinase activity.

We must also point out the high specific activity of the pituitary enzyme: 0.3–0.5 unit/mg post-mitochondrial protein; as a matter of fact, it is several times higher than that of brain and liver. Furthermore, it is located in the range of reported activities from highly proliferating tissues. The following activities have been recalculated from literature (nmoles dTMP phosphorylated per min per mg protein): 0.005 in 100 000 $\times$ *g* supernatant of 24-h regenerating liver[17], 0.02 in 100 000 $\times$ *g* supernatant of rat liver 39 h after recovery of protein malnutrition[2], 0.001 in whole homogenate from mouse parotid gland after stimulation of DNA synthesis by isoproterenol[18], 0.033 in extract of Landschütz Ascites tumor cells[19], 0.3 in 10 000 $\times$ *g* supernatant of calf thymus[20], 1.6 from mouse ascites hepatoma[3] and 3.0 in high speed supernatant of *Rana pipiens* embryo homogenates[15].

We have already shown that pituitary development goes through 3 steps[6,7], the second one being characterized by a strong increase of total protein/DNA ratio (hypertrophy). As there is no important DNA synthesis at that time, one can wonder

why dTMP kinase molecules are synthesized (or activated) during the hypertrophy phase, unless we assign some important, but yet unknown, role to thymidylic nucleotides during the prepuberty period. Further experiments should be performed to elucidate this possibility.

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