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Partial purification and some properties of a nucleoside phosphotransferase of chick embryos

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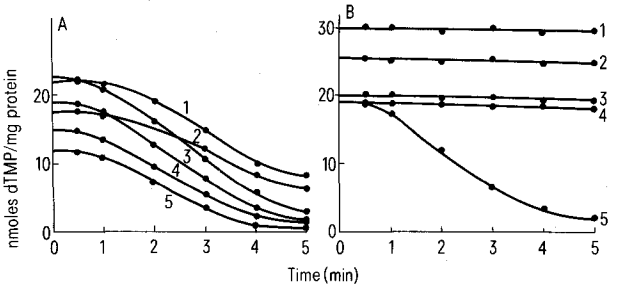
Summary. A nucleoside phosphotransferase purified about 40fold from chick embryos utilizes efficiently as phosphate donors deoxyribonucleoside and pyrimidine ribonucleoside monophosphates, whereas the pyrimidine deoxyribonucleoside appear to be the preferred acceptors of phosphate. The enzyme is very unstable to heat, dilution and dialysis. A marked enhancement in the stability is caused by nucleotides and it seems associated with the formation of an aggregated state of the protein.

Previously[1] we have found in the retina of the chick embryos 2 different non-specific forms of nucleoside phosphotransferase which are able to phosphorylate thymidine, and we have hypothesized that these forms could be an expression of the same enzyme at different aggregation states. We describe now some properties of a nucleoside phosphotransferase activity purified about 40fold from 12-day-old chick embryos.

Methods. 50 chick embryos were homogenized with 300 ml of 5 mM tris-HCl buffer pH 8.0. The homogenate was centrifuged at 105,000 × g for 30 min and the supernatant was collected. Protamine step was performed by 2 successive additions of 1% protamine sulfate to this supernatant. At first 6.25 ml/g of protein were added and the resulting mixture was stirred and centrifuged, and the precipitate discarded. Successively a new aliquot of 1% protamine sulfate (16.6 ml/g of protein) was added and the precipitate was homogenized with 50 ml of 0.2 M KH_2PO_4, pH 8.0 (protamine I). This homogenate was centrifuged and the supernatant collected. Proteins were then precipitated by solid ammonium sulfate to 30%, resuspended in 40 ml of 5 mM tris-HCl pH 8.0 and dialyzed for 12 h at 4 °C against 400 ml of the same buffer. After dialysis, 4 ml of 1% protamine sulfate were added and the precipitate was homogenized with 20 ml of 0.2 M KH_2PO_4 (protamine II). After centrifugation, solid ammonium sulfate to 50% saturation was added to supernatant. The precipitate obtained was resuspended in 10 ml of tris-HCl pH 8.0 and dialyzed for 12 h at 4 °C against 50 ml of the same buffer. This dialyzate (ammonium sulfate II) was generally utilized for the incubation samples.

The standard reaction mixture contained, in a final volume of 500 µl, 40 mM tris-HCl buffer pH 8.8; 20 µM (0.5 µCi) (Me-^{3}H)thymidine; 5 mM nucleotide (phosphate donor); 10 µl of enzyme or 50 µl of homogenate or 105,000 × g supernatant. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 0.2 ml of 10% trichloroacetic acid. The nucleoside phosphotransferase activity was evaluated as previously reported[1]. Protein was estimat-



Stability of nucleoside phosphotransferase at 37 °C. *A* Stability in relation to the phosphate donor employed. For each sample about 90 µg of purified enzyme were preincubated at 37 °C in a volume of 0.3 ml with 2.5 µmoles of $MgCl_2$ and 20 µmoles of tris-HCl buffer pH 8.8. At the time intervals indicated, 10 nmoles were (0.5 µCi) of (Me-^{3}H) thymidine and 2.5 µmoles of phosphate donor were added and the incubation was carried out at 37 °C for 30 min in a final volume of 0.5 ml (1, d-UMP; 2, d-TMP; 3, d-AMP; 4, UMP; 5, d-GMP; 6, CMP). *B* Protective effects of various nucleotides. The preincubation mixture as in *A*, except that a nucleotide protector was added. At the indicated time intervals, 10 nmoles (0.5 µCi) of (Me-^{3}H) thymidine and 2.5 µmoles of UMP were added and the incubation was carried out at 37 °C for 30 min (1, addition of 1 nmole of d-TTP; 2, 1 nmole of UDP; 3, 2 nmoles of d-UMP; 4, 2 nmoles of d-TMP; 5, no addition).

Table 1. Purification of the nucleoside phosphotransferase of chick embryo. For these experiments UMP was utilized as phosphate donor

Fraction	ml	Protein (mg)	Total activity (units)*	Units/mg of protein	Phosphotransferase
Homogenate	450	9540	4333	0.454	-
105,000× g supernatant	310	2720	3209	1.179	2.59
Protamine I	50	760	2644	3.478	7.66
Ammonium sulfate I	40	195	2277	11.676	25.71
Protamine II	20	110	1834	16.672	36.72
Ammonium sulfate II	10	90	1715	19.055	41.97

*1 unit of enzyme is defined as that amount which, in our experimental conditions, converts 1 nmole of thymidine in thymidilic acid.

Table 2. Effect of various phosphate donors on the nucleoside phosphotransferase activity measured with the 105,000× g supernatant (A) or with the purified enzyme (B)

Phosphate donor (5 mM)	A	B
	(nmoles of d-TMP/mg of protein)	
AMP	0.45±0.04	0.30±0.03
ADP	0.40±0.04	no activity
ATP	0.35±0.03	no activity
GMP	0.60±0.05	0.56±0.05
GDP	0.55±0.06	no activity
GTP	0.48±0.06	no activity
CMP	0.74±0.09	11.95±2.12
CTP	0.21±0.02	2.36±0.25
UMP	1.18±0.10	19.06±2.20
UDP	1.04±0.12	13.18±1.50
UTP	0.52±0.06	5.88±0.60
d-AMP	1.39±0.12	22.56±2.80
d-GMP	0.92±0.10	15.08±1.65
d-CMP	1.15±0.20	14.08±1.30
d-CTP	0.78±0.08	9.36±1.05
d-TMP	1.33±0.15	16.68±1.95
d-TTP	0.39±0.05	4.47±0.55
d-UMP	1.79±0.20	22.86±2.45

Values are the means of 7 separate experiments ± SE.

Table 3. Phosphate transfer to various nucleosides. For these experiments UMP was utilized as phosphate donor

Nucleosides	Nucleotide formed (nmoles/mg of protein)
Guanosine	0.35±0.03
Cytidine	2.20±0.20
Uridine	3.15±0.38
d-Cytidine	12.83±1.40
d-Thymidine	19.25±1.86
d-Uridine	16.00±1.55

Data are the means of 7 separate experiments ± SE.

Table 4. Effects of various phosphate donors on the activity of the enzymatic forms A and B. In these experiments Tris-HCl buffers pH 8.8 or 7.6 were used respectively in the incubation samples for form A and B

Substrates	Form A	Form B
	(nmoles of d-TMP/mg of protein)	
AMP	0.61±0.08	1.02±0.12
GMP	0.92±0.10	1.30±0.15
CMP	25.20±2.85	0.80±0.09
UMP	40.20±4.95	1.38±0.18
UDP	25.10±2.85	1.20±0.18
UTP	12.05±1.35	0.66±0.08
d-AMP	44.30±5.20	1.50±0.17
d-GMP	30.70±3.55	1.05±0.12
d-CMP	27.50±3.05	1.37±0.15
d-UMP	44.10±4.86	2.32±0.28
d-TMP	35.20±3.92	1.68±0.20

Values are the means of 7 separate experiments ± SE.

ed by the method of Lowry et al.[2] by using bovine serum albumine as standard.

Results and discussion. The purification procedure reported in table 1 has been followed 10 times with excellent reproducibility. The highest specific activity obtained was approx. 19 units/mg protein, but values much higher can be produced by employing a stabilizing agent.

As shown in table 2, the phosphotransferase activity is capable of phosphorylating thymidine by using various nucleotides as phosphate donors. The reaction is more efficient with the nucleoside monophosphates than di- or triphosphate forms. With the purified enzyme, all the deoxyribonucleotides and the pyrimidine ribonucleotides tested were active as phosphate donors. In contrast, only a little activity was shown for the purine ribonucleotide monophosphates. The observation that ATP is ineffective in this case as phosphate donor allows us to differentiate nucleoside phosphotransferase from thymidine kinase.

The capacity of various nucleosides to participate as acceptors in the phosphotransferase reaction was measured with UMP as phosphate donor. The results reported in table 3 indicate that the preferred acceptors of phosphate appear to be the pyrimidine deoxyribonucleosides (d-thymidine, d-uridine, d-cytidine).

The nucleoside phosphotransferase has a very unstable activity, in fact the enzyme was quickly inactivated in diluted solutions also at room temperature (15 °C). After dialysis for 24 h at 4 °C against 100 vol. of 5 mM tris-HCl buffer pH 7.6, about 3% of the activity was recovered.

Further, as shown in the figure, A, a marked decrease in the phosphotransferase activity was observed, when the enzyme was preincubated at 37 °C for 5 min. The inactivation was lower when d-UMP or TMP were utilized as phosphate donors. For this reason, the ratio V_{tmp}/V_{ump} increases in relation to the preincubation time. A marked enhancement in the stability of the enzyme is caused by nucleotides. In fact, the enzyme was not inactivated when 1 nmole of d-TTP or UDP, or 2 nmoles of d-UMP or TMP were added in the preincubation mixture (figure 1, B). For the experiments reported in the figure, B, UMP was utilized as phosphate donor but identical effects were obtained by using as substrate CMP or d-AMP or d-GMP. This marked enhancement in the stability is associated with the formation of an aggregate state of the protein. In fact, when the nucleoside phosphotransferase was submitted to gel filtration on a Sephadex G-200 column[1], and the column was eluted with 5 mM tris-HCl buffer pH 8.0 containing 50 μm UDP, the elution of a single enzymatic peak (form A) at high mol. wt was observed with a recovery of the activity equal to about 90%. The pH optimum of this form A was about 8.8. In the absence of a nucleotide protector, also a single peak (form B) was eluted but at lower mol. wt and with a recovery equal only to 3%. This form B has a pH optimum about 7.6. The activity of the enzymatic form A and B in relation to different phosphate donors is reported in table 4. Much lower values were obtained with form B for all the substrates tested, except for purine ribonucleo-

side monophosphates (AMP and GMP). Consequently, for form B the activity measured with UMP as phosphate donor (V_{ump}) was little different to that measured with AMP (V_{amp}). Therefore, the ratio V_{ump}/V_{amp} was in this case very low (1.35), whereas it reached a much higher value for form A (65.9).

It appears however that form A represents a very active and aggregate state of nucleoside phosphotransferase, whereas the form B corresponds to a disaggregate and much less active state. Therefore the value of the ratio V_{ump}/V_{amp} may provide information on the state of aggregation of the enzyme. Thus low values of this ratio seem to indicate a clear preponderance of the disaggregate form B.

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Age-dependent response of lactate dehydrogenase of pituitary of rat to testosterone

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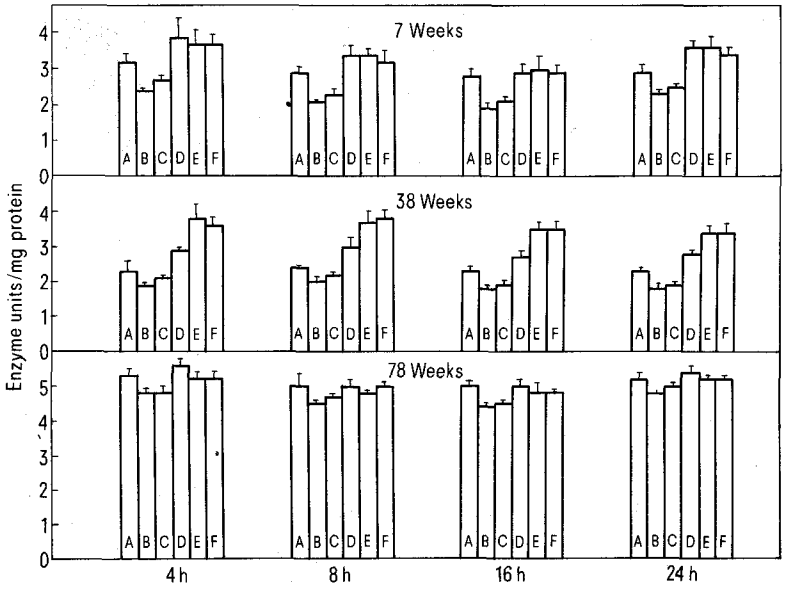
Summary. The effects of time and various doses of testosterone on the responsiveness of lactate dehydrogenase of pituitary of 7-, 38- and 78-week-old rats were studied. The activity of the enzyme increases in 78-week-old rats. Castration decreases the enzyme activity at all ages. Maximum increase in the enzyme activity is seen with 50 and 100 µg of testosterone 4 h after administration of hormone to castrated rats. No further time and dose-dependent effect is observed. The magnitude of increase for the enzyme is higher at the age of 38 weeks and decreases in 78-week-old rats.

Alterations in qualitative and quantiative nature of enzymes may be one of the factors contributing to the process of senescence[2-5]. The changes in the levels of enzymes can be modulated by the factors like hormones. Age-dependent modulation of certain enzymes of the rat have been reported[6-8]. The ability to stimulate an adaptive change in the activity of a large number of enzymes is dependent on dose of stimulant, duration of treatment, tissue and age of the animal[9]. Everitt[10] proposed that the process of aging is regulated by hypothalamic-pituitary-peripheral endocrine axis. We report here the time, dose as well as age-dependent changes in the responsiveness of the enzyme, lactate dehydrogenase (LDH; E.C. 1.1.1.27) of pituitary to testosterone. This enzyme catalyses the reversible reaction of pyruvate and lactate of glycolytic pathway.

Materials and methods. Wistar strain albino rats were maintained under standard laboratory conditions; 7-, 38- and 78-week-old male rats were used. The rats of each age were divided into 6 sets each with 30-36 rats. 1st set served as normal rats. The remaining 5 sets of each age were castrated bilaterally under ether anaesthesia. They were kept for 21 days. On the 22nd day, the rats of 2nd set were administered with vehicle solution. This group served as control to the hormone-treated rats. The rats of 3, 4, 5 and 6 sets were given i.p. with 10, 50, 100 and 200 µg testosterone/100 g b.wt respectively at 16.00 h. The hormone was dissolved in 10% ethanol-normal saline solution. The rats of each set were killed by cervical dislocation at 4, 8, 16 and 24 h after administration of the hormone; 5-6 rats were sacrificed from each set at the time mentioned above. The pituitary was taken out and 2% homogenate was prepared with 0.1 M phosphate buffer, pH 7.4 in a cold room. The homogenate was centrifuged for 30 min at 14,000 × g at 0 °C. The supernatant was used for spectrophotometric assay of LDH[11]. The enzyme activity was expressed as units/mg protein (specific activity) after determining the protein content in the supernatant[12].

The assay mixtures of normal and castrated rats were incubated with testosterone (10^{-7} M, 10^{-6} M, 10^{-5} M) to find out whether the effects of this hormone administration on



Time, dose and age-dependent response of LDH of pituitary to testosterone. *A* normal rats; *B* castrated rats (C); *C* C + testosterone 10 µg; *D* C + testosterone 50 µg; *E* C + testosterone 100 µg; *F* C + testosterone 200 µg. Bars indicate SD.