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BIOCHEMISTRY OF THE DEVELOPING RAT BRAIN

I. SOLUBLE ENZYMES IN ISOLATED NEONATAL BRAIN NUCLEI*

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SUMMARY

A procedure is described for the isolation of neonatal rat-brain nuclei free of debris using a Triton X-100–sucrose medium for homogenization to facilitate removal of erythrocytes and cytoplasmic organelles. The isolated nuclei swell in water without rupture indicating that the nuclear membranes were intact. To rupture these nuclei it was necessary to use 1 M Tris buffer.

Assays of the nuclear extracts established the presence of the glycolytic enzymes. Thus, generation of ATP from ADP occurs by the action of triose phosphate dehydrogenase and pyruvate kinase. Adenylate kinase and ATPase were also present in these extracts, which suggests that they act as a means for maintaining the required levels of adenosine mono- and polyphosphates in the nuclei.

NADP-isocitric dehydrogenase and malic dehydrogenase were also found in the nuclear extracts, however, their role in nuclear metabolism is unknown.

INTRODUCTION

In the course of our studies on the enzyme changes in the developing rat brain, it was noted that rat-brain nuclei have not been previously isolated hence no enzymic and metabolic data were available. Reports on the generation of ATP in isolated thymus nuclei indicated a strict aerobic dependence[1] for this synthesis, while isolated nuclei from ox brain, rat liver and pig kidney generate ATP solely by anaerobic glycolysis[2]. In order to determine the metabolic route responsible for the generation of ATP, neonatal rat-brain nuclei were isolated and the soluble enzymes from these nuclei were investigated.

This report describes the procedure for isolating neonatal brain nuclei and summarizes the enzyme activities found in the soluble nuclear fraction.

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METHODS

Isolation procedure

Twenty-four to forty newborn Sprague–Dawley rats were decapitated in groups of four and the brains were quickly removed free from the spinal cord just below the medulla, exclusive of the olfactory bulbs. The brains, each weighing 0.23 g, were homogenized in a glass homogenizer with a Teflon pestle in 9 volumes of medium/g of tissue. All operations were performed at 0–4°. The medium contained 0.40 M sucrose and 0.003 M $MgCl_2$. After homogenization, 0.25 ml of a 10% solution of Triton X-100 (isooctylphenoxypolyethoxyethanol, Rohm and Haas Company, Philadelphia, Pa.) was added to each 10 ml of homogenate, to a final Triton concentration of 0.25 %. This mixture was homogenized again, and then allowed to stand in the ice-bath for 20 min. Subsequently, the mixture was centrifuged at 750 × *g* for 15 min and the supernatant was removed and discarded. The white pellet was resuspended in the 0.2 M sucrose and 0.003 M $MgCl_2$ in a total volume equal to the original homogenate, allowed to stand for 5 min and then centrifuged at 750 × *g* for 15 min. The supernatant was removed and this treatment was repeated once more. This yielded a white pellet containing whole nuclei free from cytoplasmic debris.

Rupture of nuclei

The nuclear pellet from 14 rats was suspended in 0.8 ml of 1 M Tris buffer (pH 7.4), homogenized manually, and then diluted with 3 volumes of cold glass-distilled water to a final concentration of 0.25 M Tris. To facilitate complete rupture of the nuclei, the mixture was further subjected to manual homogenization. Complete rupture of nuclei was established by microscopic examination of wet smears stained with Janus green B.

The white, viscous mixture of ruptured nuclei was centrifuged at 12000 × *g* for 1 h and the clear supernatant was separated from the gelatinous residue. This supernatant was used for evaluation of soluble nuclear enzymes.

The procedure used for the isolation and rupture of nuclei is schematically represented in Fig. 1.

Enzyme determinations

All enzymic activities in the nuclear extracts were determined spectrophotometrically by recording the rate of change in the oxidation or reduction of pyridine nucleotides at 340 mμ. A Beckman recording double-beam spectrophotometer was used for these assays in which the sample cuvettes were maintained at 30° by circulating water. The final volume of the assay mixtures ranged from 0.8 to 1.0 ml, and the blanks contained all the components of the sample except the pyridine nucleotide. For each assay, preliminary controls were tested to standardize the procedure. The assay components were mixed quickly in a 1-ml quartz cell (1.0-cm light path) then immediately placed in the spectrophotometer, and the recordings were made over periods of 5–15 min. The results are reported as specific activities of the enzymes ± standard deviations of the mean as mμmoles of pyridine nucleotide reduced or oxidized/min/mg of protein. Enzymes added as coupling systems for assay of nuclear enzymes were purchased from the Calbiochem, Inc., Los Angeles, Calif. Crystalline DNAase was purchased from Worthington Biochemical Corp., Freehold, N.J.

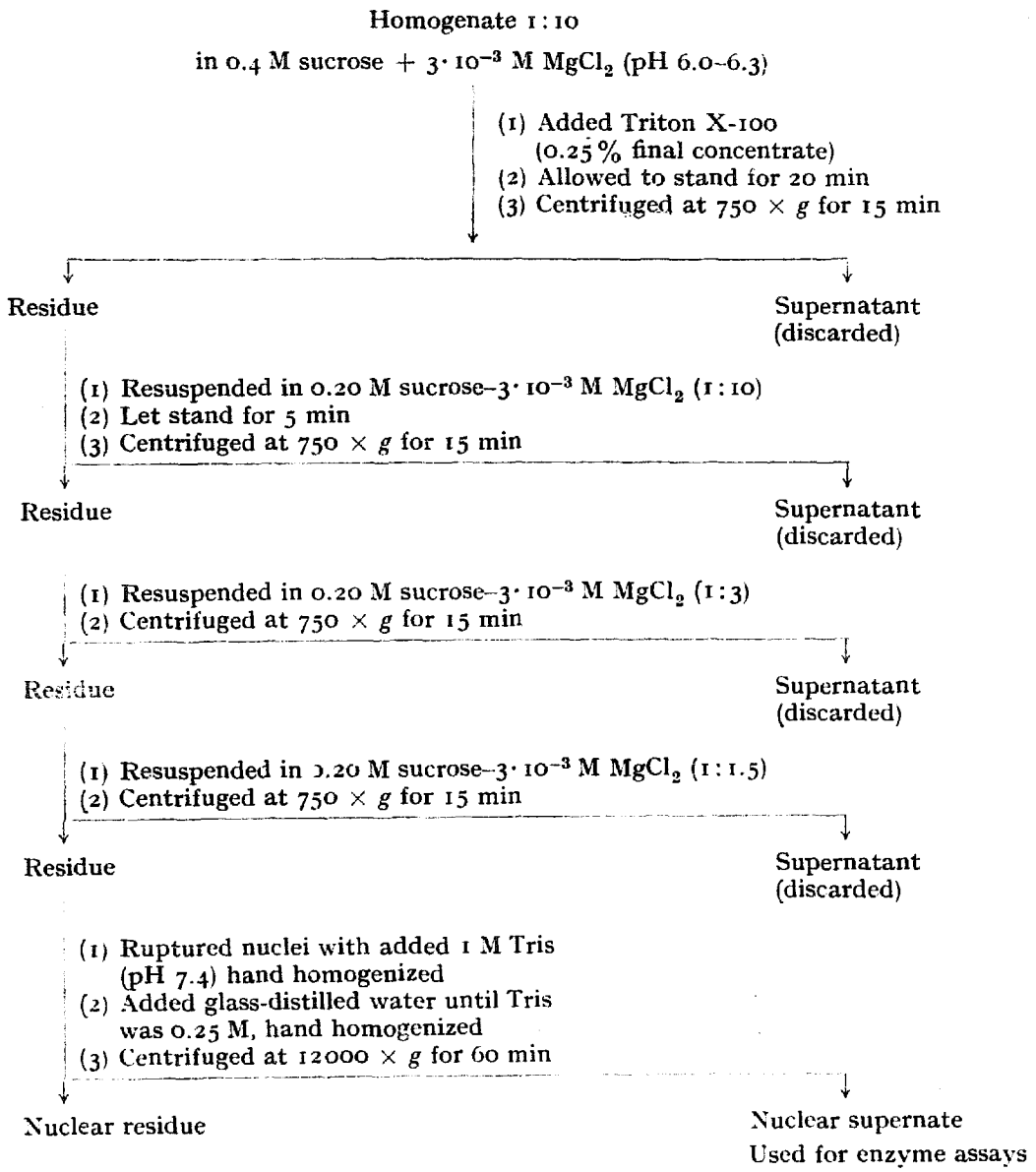

Fig. 1. Schematic outline of the procedure for isolation of nuclei and nuclear extract.

Protein was determined by the procedure of LOWRY *et al.*,[3] or by the change in absorbancy at 215–225 mμ according to WADDELL[4].

Enolase

The method used is similar to that described by BUCHER AND PFLEIDERER[5] for assay of pyruvate kinase. The reaction mixture contained 40 mM triethanolamine buffer (pH 7.4), 55 mM KCl, 6 mM $MgCl_2$, 0.35 mM ADP, 0.6 mM DL-2-phosphoglyceric acid, 125 μM NADH, lactic dehydrogenase and pyruvate kinase as coupling enzymes, and the nuclear extract.

Pyruvate kinase

This assay medium was the same as the one described above for enolase except pyruvate kinase was omitted and the substrate was 1.2 mM phosphoenolpyruvate.

Lactic dehydrogenase

The method used for this determination is essentially that described by KORNBERG[6]. The medium contained 40 mM triethanolamine (pH 7.4), 0.65 mM sodium pyruvate, 120 μM NADH, and the nuclear extract.

Hexokinase[7]

The medium used for this analysis contained 3 mM glucose, 4.5 mM ATP, 40 mM triethanolamine buffer (pH 7.4), 8 mM KCl, 8 mM $MgCl_2$, 1.5 mM EDTA, 0.3 mM NADP, glucose-6-phosphate dehydrogenase, and the nuclear extract.

Aldolase

The method for this assay closely followed the procedure described by BARANOWSKI AND NIEDERLAND[8] with minor modifications. The reaction mixture contained 8 mM fructose 1,6-diphosphate, 2.5 mM ammonium phosphate, 1.6 mM $MgCl_2$, 5 mM cysteine·HCl, 0.25 mM NADH, a mixture of α-glycerophosphate dehydrogenase and triose phosphate isomerase as the coupling enzyme, and nuclear extract.

Triose phosphate dehydrogenase

This enzyme was assayed by the procedure described by BEISENHERZ *et al.*[9] and modified by WU AND RACKER[10] with the exception that triethanolamine buffer was used along with EDTA. The reaction mixture consisted of 40 mM triethanolamine buffer (pH 7.4), 8 mM $MgCl_2$, 2 mM EDTA, 5 mM ATP, 8 mM potassium 3-phosphoglycerate, 0.25 mM NADH, phosphoglycerate kinase and the nuclear extract.

Adenosine triphosphatase

The assay mixture is similar to that used for the determination of pyruvate kinase. This solution contained 40 mM Tris buffer (pH 7.3), 1.2 mM phosphoenolpyruvate, 2 mM $MgCl_2$, 20 mM KCl, 1.5 mM ATP, 0.25 mM NADH, with pyruvate kinase and lactic dehydrogenase as coupling enzymes and the nuclear extract. The blank consisted of the above mixture but without ATP.

Adenylate kinase and adenosine triphosphatase

The determination of adenylate kinase activity was made by the addition of 5-adenylic acid to the reaction mixture used for ATPase assay. The final concentration of adenylate was 1.5 mM. This method gave the combined activities of adenylate kinase and ATPase.

It was noted that addition of adenylate to the reaction mixture increased the rate of NADH disappearance which indicated that adenylate kinase mediated the reaction AMP + ATP $\longrightarrow$ 2 ADP. The control tests gave the following results: (a) Without addition of ATP or AMP, the absorbancy did not decrease for 30 min. (b) When AMP was added (excluding ATP) there was no decrease in absorbancy for 30 min.

NADP-isocitric dehydrogenase[11]

For this assay, the reaction mixture contained 180 mM Tris (pH 7.4), 15 mM triethanolamine (pH 7.4), 2 mM $MgCl_2$, 20 mM KCl, 1.5 mM $MnCl_2$, 250 μM NADP, 1.5 mM sodium isocitrate and the nuclear extract.

NAD-malic dehydrogenase[12]

The medium for this test contained 180 mM Tris buffer (pH 7.4), 15 mM triethanolamine (pH 7.4), 2 mM $MgCl_2$, 20 mM KCl, 250 μM NADH, 1.5 mM sodium oxaloacetate and nuclear extract.

RESULTS

Neonatal brain homogenates prepared in sucrose without Triton always contained numerous erythrocytes which remained intact throughout the nuclei isolation procedure. Thus, the resulting nuclear pellet was colored pink or red. Homogenates prepared in Triton medium were entirely free of erythrocytes as well as intact glia or neurons. Fig. 2 is a microphotograph of such a homogenate in which the nuclei have been stained with Janus green B and photographed as a wet smear. Nuclei isolated from these homogenates were free of any cytoplasmic inclusions, erythrocytes, and dissolved hemoglobin hence the final nuclear pellet was white in color and the nuclei appeared as shown in Figs. 3 and 4. Here too, the nuclei were stained with Janus green B and photographed as wet smears. All attempts to prepare "fixed" (dried) nuclear slides yielded ruptured nuclei.

Attempts to isolate nuclei from young (21 days) and mature (90 days) rat brains by the procedure outlined in Fig. 1, yielded intact nuclei heavily contaminated with cell debris (myelin) and fat globules.

When the isolated nuclei from neonatal, young, and mature brains, were suspended in distilled water, they did not rupture but became distended and more

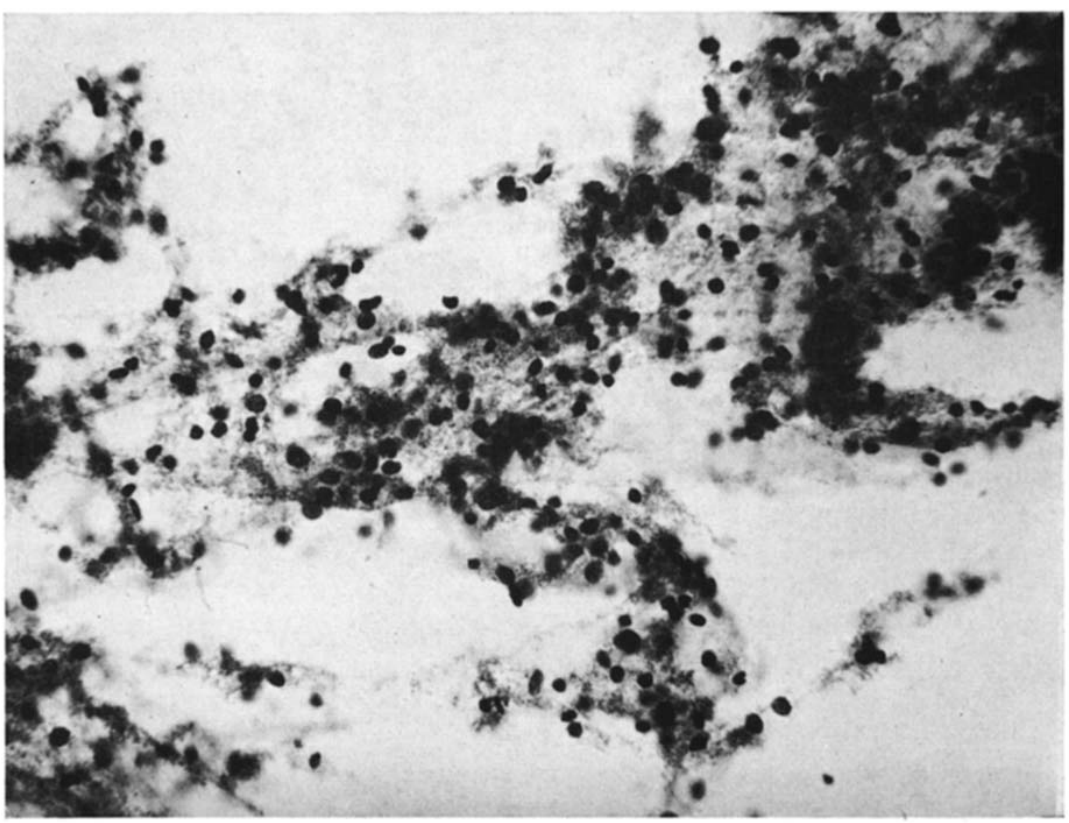

Fig. 2. Neonatal rat-brain homogenate stained with Janus green B (× 196).

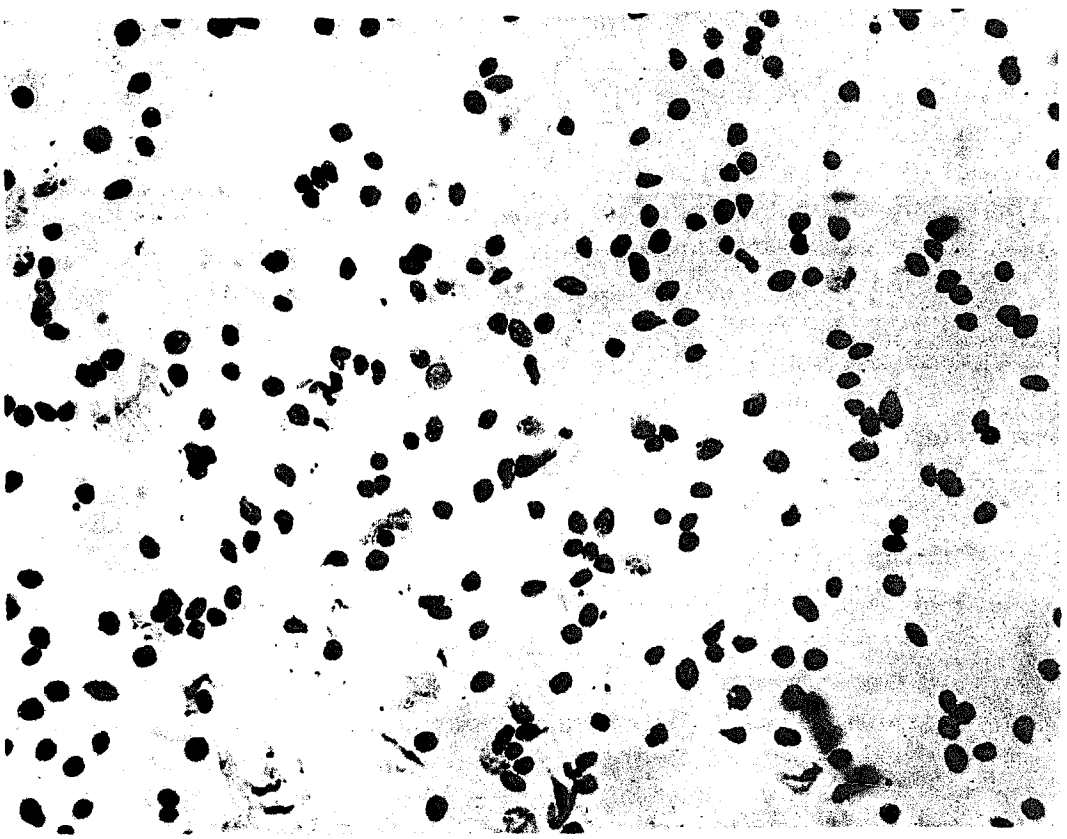

Fig. 3. Isolated neonatal rat-brain nuclei stained with Janus green B (× 252).

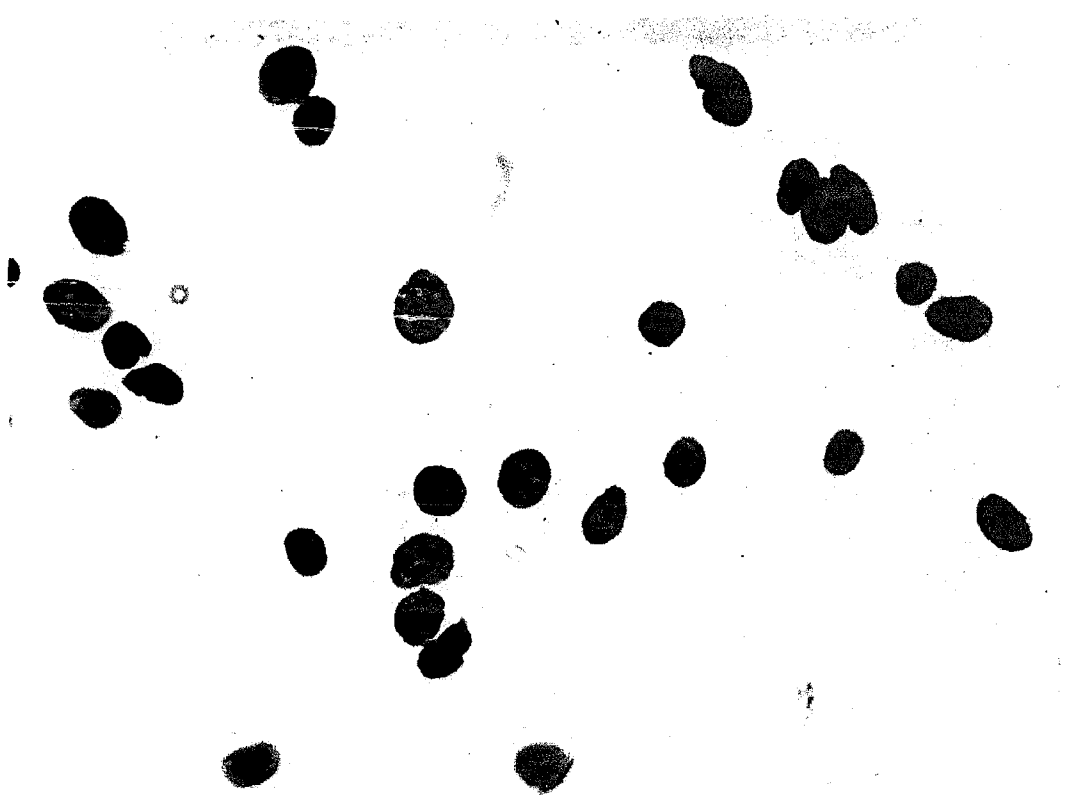

Fig. 4. Isolated neonatal rat-brain nuclei stained with Janus green B (× 599).

perfectly spherical. In order to rupture these nuclei, it was necessary to suspend them in 1 M Tris buffer (pH 7.4). This treatment ruptured all the nuclei and produced a viscous mixture. After dilution with distilled water to a final Tris concentration of 0.25 M and centrifugation at 12000 × *g* for 1 h, this mixture gave a clear non-viscous supernatant (nuclear extract) and a gelatinous white pellet.

The gelatinous nuclear pellet was tested for DNA by treatment with crystalline DNAase. Within 20 min at room temperature, the viscosity of the mixture was drastically reduced indicating depolymerization of DNA. A white precipitate was present in this mixture which easily sedimented at 2000 × *g*. Assays for enzyme activities in the supernatant from this DNAase treated nuclear residue, showed only a small amount of activity, presumably due to carry-over of enzymes from the original nuclear extract.

TABLE I

ENZYME ACTIVITIES IN THE NUCLEAR EXTRACT FROM NEONATAL BRAIN

Enzyme	*Number of expts.*	*Number of assays*	*Specific activity**
Hexokinase	3	9	33.0 ± 2.6
Aldolase	3	9	57.1 ± 5.1
Enolase	3	9	1.67 ± 0.2
Pyruvate kinase	2	6	219 ± 1.3
Lactic acid dehydrogenase	2	6	29.5 ± 1.3
Triose phosphate dehydrogenase	3	9	215 ± 13
NADP-isocitric dehydrogenase	2	6	2.41 ± 0.18
NAD-malic dehydrogenase	2	6	34.0 ± 2.5
ATPase	2	6	5.60 ± 0.35
ATPase and adenylate kinase	2	6	12.1 ± 0.5

* Specific activity expressed in mμmoles of pyridine nucleotide reduced or oxidized/min/mg protein.

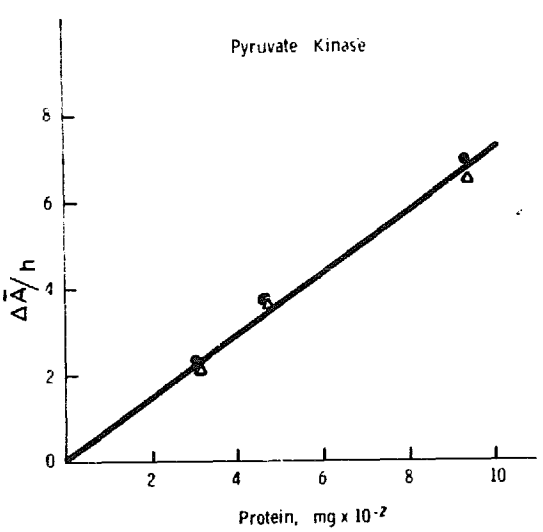

Fig. 5. Pyruvate kinase activity in neonatal rat-brain nuclear extracts from two independent experiments illustrating replication of results.

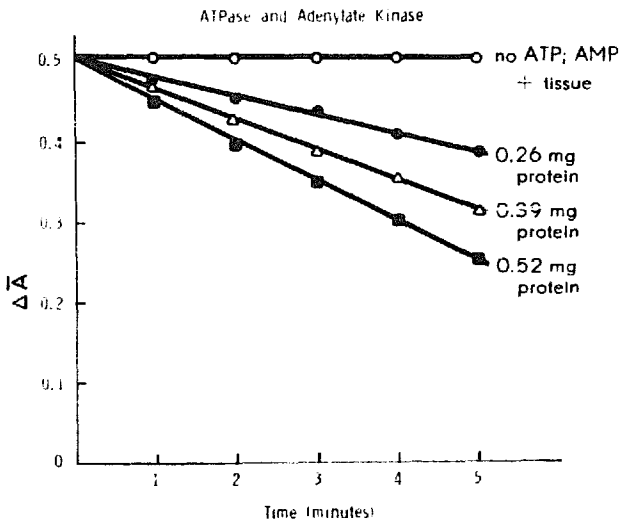

Fig. 6. Rate changes of the combined activities of adenosine triphosphatase and adenylate kinase, in the neonatal rat brain nuclear extracts, with changes in protein levels.

The enzyme activities detected and measured in the nuclear extract are listed in Table I. With a few exceptions, almost all the glycolytic enzymes were present in this extract. Also present were ATPase, adenylate kinase, NADP-isocitric dehydrogenase and malic dehydrogenase (Table I). The levels of the enzyme activities varied; thus triose phosphate dehydrogenase and pyruvate kinase were the most active, and enolase was the least active.

Replication of results from enzyme assays is examplified in Fig. 5. Pyruvate kinase activities are plotted from two independent experiments, and the results are identical. The linear relationship between enzyme activity and enzyme concentration (increasing amounts of nuclear extract) is illustrated in both Figs. 5 and 6. In the latter the combined activities of ATPase and adenylate kinase double as the concentration of nuclear extract is doubled.

DISCUSSION

The isolation procedure for rat-brain nuclei, described in this study, yielded nuclei not only free from cellular debris, but with intact nuclear membranes. Swelling of these nuclei in water is evidence of this fact. This also indicates that Triton X-100 very likely affected the nuclear membrane so that it was not readily ruptured by osmotic swelling. It was essential to use this non-ionic detergent in order to lyse the erythrocytes sequestered in the isolated brain, and facilitate removal of erythrocyte glycolytic enzymes. Triton also facilitated rupture of mitochondria and other cytoplasmic inclusions, thus permitting isolation of neonatal nuclei free from debris. However the concentrations of Triton used in these homogenates were ineffective in solubilizing all the cellular components in brain where myelin formation had started and had progressed.

When isolated nuclei were obtained, the possibility was considered, that some of the cytoplasmic enzymes may adsorb to the nuclear membrane and subsequent washings may not dislodge them completely. With this in mind, it was believed that rupture of the nuclei and examination of the enzyme constituents in the nuclear extract would, to a degree, minimize the effect of any adsorbed enzymes.

In addition, using nuclear extracts avoided any complications that may stem from differential permeability of substrates through intact nuclear membrane, particularly when the membrane was altered by Triton.

The enzymes found in the nuclear extracts (Table I) can generate ATP by glycolysis, as long as glucose, some ATP and ADP are available. The presence of ATPase will prevent accumulation of ATP and insures availability of ADP from ATP. Also, adenylate kinase serves to prevent excessive accumulation of either ADP, AMP or ATP. Thus the brain nuclei can generate ATP and keep the necessary quantitative balance between the adenosine phosphates to maintain active glycolysis. The function of NADP-isocitric dehydrogenase and malic dehydrogenase in the nuclei is at present not clear, but it is likely that these enzymes participate in maintaining definitive levels of oxidized or reduced pyridine nucleotides for nuclear functions.

The results obtained in the present study with isolated brain nuclei conform to the findings reported by SIEBERT[2] with ox brain, pig heart and rat liver in which glycolysis was mainly responsible for ATP generation.

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