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CHARACTERIZATION OF AN IN VITRO PROTEIN SYNTHESIS SYSTEM FROM THE CEREBRUM, CEREBELLUM, AND OPTIC LOBES OF CHICK BRAIN

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ABSTRACT

A highly active in vitro protein synthesizing system has been obtained from the cerebrum, cerebellum, and optic lobes of chick brain. The post-mitochondrial supernatant of each region was used for in vitro protein synthesis after Sephadex G-25 chromatography and was found to be most active when incubated at 37°C. Optimum conditions for the three regions of chick brain were similar and are as follows: 8.5 mM Mg^{++}, 120 mM K^{+}, pH 7.3 (at 37°C), 3 mM ATP, 0.75 mM GTP, and a mixture of L-amino acids containing 20 μM of each amino acid. It was found that ammonium and rubidium ions could substitute for K^{+} while high concentrations of lithium and sodium ions could not. Under optimum conditions, the level of incorporation by the cerebellum or optic lobes was consistently 20 tp 40% less than the cerebrum.

INTRODUCTION

In recent years interest in the biochemical study of the brain has mushroomed. One biochemical process often studied is protein synthesis. The brain is a rather unique tissue in that its development and maturation occur to a significant extent before birth; therefore, it is often important to study the brain during embryonic development. All studies on in vitro protein synthesis in brain tissue have been limited to mammalian systems such as rats (Campbell, 1966; Clouet, 1966; Campagnoni, 1967; Mahler, 1968; Zomzely, 1966; Zomzely-Neurath, 1972), mice (Lerner, 1970), and rabbits (Stenzel, 1966). Embryonic studies in these systems are difficult and often extremely limited. The chick provides a unique system where embryonic brain tissue can be easily obtained at various stages of development and manipulated. This report describes the first characterization of an in vitro protein synthesis system from chick brain and a comparison of protein synthetic activities of different regions of the chick brain.

MATERIALS AND METHODS

Animals. Fertilized White Leghorn eggs were obtained from the University of Illinois Poultry Farm and incubated at 37.5°C in a relative humidity of 52%. The eggs were placed on racks tilted at 45° and turned 12 times daily. Under these conditions, the hatchability was found to be 80-90%. After hatching, chicks were transferred to room temperature and fed ad libitum a commercial feed containing 18% crude protein, 3% crude fat, and 6% crude fiber. In addition, the feed contained the drugs amprolium (0.125%) and ethopabate (0.0004%). For this study brains from 16-day-old embryos to 2-day-old chicks were used. These ages were chosen because protein synthesis was found to be high during this period.

Materials. ATP, GTP, L-amino acids, creatine phosphate, creatine-phosphokinase (E.C. 2.7.3.2), glutathione, sucrose, Sephadex G-25, N-2-hydroxy-ethyl-piperazine-N'-2-ethane sulfonic acid (HEPES) were obtained from Sigma Chemical Company, St. Louis, Missouri. Uniformly labeled L-^{14}C-valine (specific activity 210-252 mCi/m mole) was purchased from New England Nuclear, Boston, Mass.

Preparation of post-mitochondrial supernatant. Embryos or chicks were decapitated, and the cerebrum, cerebellum, and optic lobes were isolated and kept on ice separately. Samples of each were added to 2 volumes of homogenization buffer (adjusted to pH 7.3 at room temperature with 24 mM KOH) which contained 50 mM HEPES, 8.5 mM $MgCl_2$, 96 mM KCl, 0.25 M sucrose and 3 mM glutathione. The presence of glutathione in the homogenization buffer was found to increase protein synthesis 10-20%. Samples were then homogenized with 4-5 passes of a Potter-Elvehjem tissue grinder, the homogenates were centrifuged at 20,000 xg for 8 min at 4°C, and the resulting post-mitochondrial supernatant (PMS) was saved. The PMS of the three regions was prepared for amino acid incorporation by passing 1.35 ml over a 1.5 x 13 cm Sephadex G-25 column (void volume of 15 ml) collecting the first 3-3.5 ml of PMS eluting from column.

In vitro protein synthesis. The procedure used for in vitro amino acid incorporation was similar to that previously described by Richardson (1971) for rat liver. The amino acid incorporation system consisted of a mixture of the Sephadex G-25 chromatographed PMS and incorporation medium. The latter contains ATP, GTP, L-amino acid mixture lacking L-valine, radioactive L-valine, and an energy generating system of creatine phosphate and creatine-phosphokinase.

In vitro amino acid incorporation was initiated by addition of an equal volume of chromatographed PMS to the incorporation medium. The final concentration of the components in the reaction mixture was as follows unless otherwise indicated: ATP, 3 mM; GTP, 0.75 mM; $MgCl_2$, 8.5 mM; KCl, 71 mM; L-amino acid mixture - valine, 50 μM of each amino acid; radioactive valine, 0.8 μCi/ml; HEPES, 50 mM; sucrose, 0.25 M; creatine phosphate, 14.8 mM; creatine-phosphokinase, 200 μg/ml (the pH of the final solution was adjusted to pH 7.3 at 37°C with KOH resulting in a final K^+ concentration of 120 mM). The reaction mixture was incubated at 37°C for 60 min unless otherwise indicated. The reaction was terminated by adding 0.2 ml of the reaction mixture to 2 ml of 5% trichloroacetic acid

(TCA) containing 0.25% DL-valine. Five tenths mg of bovine serum albumin was added to each sample to aid in the precipitation of protein. The resulting solutions were heated at 100°C in a water bath for 10 min to hydrolyze the radioactive valine bound to t RNA. The precipitates were collected on glass fiber filters, washed 3 times with 2 ml of 5% TCA containing 0.25% DL-valine, and dried. The radioactivity incorporated into the acid insoluble material was determined by liquid scintillation counting in a Packard Model 3003 Scintillation Spectrometer, using 0.392% 2,5-diphenyloxazole and 0.008% p-Bis-(0-methylstyryl)-benzene in toluene as the scintillation fluid. The radioactivity was expressed as disintegrations/min (DPM) of radioactive valine incorporated into acid insoluble material per µg of RNA in PMS used for incorporation. The amount of radioactive valine incorporated into acid insoluble material was directly proportional to the amount of RNA in the PMS preparation. The background radioactivities were determined by the addition of 0.1 ml of chromatographed PMS to a mixture of 0.1 ml of incorporation medium and 2 ml of 5% TCA with 0.25% DL-valine. The background ranged from 100-300 DPM while the level of radioactivity incorporated into acid insoluble material ranged from 2,000 to 10,000 DPM.

Determination of RNA. The RNA of the chromatographed PMS was determined by a modified Schmidt-Tannhauser procedure using 1.00 A_{260} = 32 µg RNA/ml (Munro, 1966).

RESULTS

The effect of temperature on the incorporation of radioactive valine into acid insoluble material by the cerebrum from chick brain is shown in Fig. 1. The highest rate of incorporation was found at 37°C and 41°C. The total radioactivity incorporated at 37°C after 60 min of incubation was 10-15% higher than incubation at 41°C. This is of interest since the body temperature of the chick is 41°C (Weiss, 1962). At 37°C, incorporation was linear for the first 15-20 min of incubation and leveled off after 45-60 min of incubation. Incorporation at 47° was greatly reduced being only 50% of that obtained at 37°C. Incubation at 25°C decreased the rate of incorporation to 40% of that observed at 37°C; however, after incubation for 85 min, the amount of incorporation observed at 25°C was similar to that obtained at 37°C. At 25°C the incorporation was linear for the first 30 to 35 min of incubation indicating that in vitro initiation of protein synthesis might be occurring in this system. Similar results were found with the cerebellum and optic lobes of the brain from embryonic stages as well as from chicks after hatching. In subsequent studies in vitro protein synthesis is expressed as the amount of radioactive valine incorporated into acid insoluble material after 1 hr of incubation at 37°C.

Ionic conditions are very important in protein synthesis. Monovalent and divalent cations affect both the structural integrity and biological function of ribosomes. Mg^{++} is essential for protein synthesis, and Fig. 2 shows that Mg^{++} requirement for in vitro protein synthesis by the cerebrum, cerebellum, and optic lobes of chick brain. The optimum concentration of Mg^{++} fell between 7 and 10 mM for all three regions of the chick brain. At Mg^{++} concentrations below 2.5 mM essentially no amino acid incorporation occurred, while concentrations above 10 mM were inhibitory. It should be noted that the different regions of the brain showed different incorporation

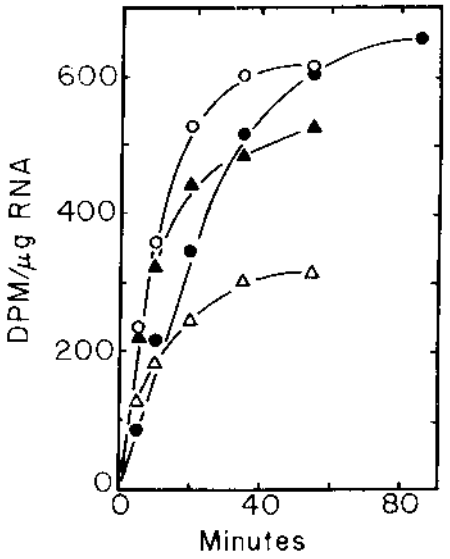

FIG. 1 -- Effect of various temperatures on in vitro amino acid incorporation by the cerebrum from embryonic chick brain. The incorporation of radioactive valine into acid insoluble material was determined as described in the methods section at 25°C (●), 37°C (○), 41°C (▲), and 47°C (△) at the various times of incubation. Each point represents the average of the duplicate samples.

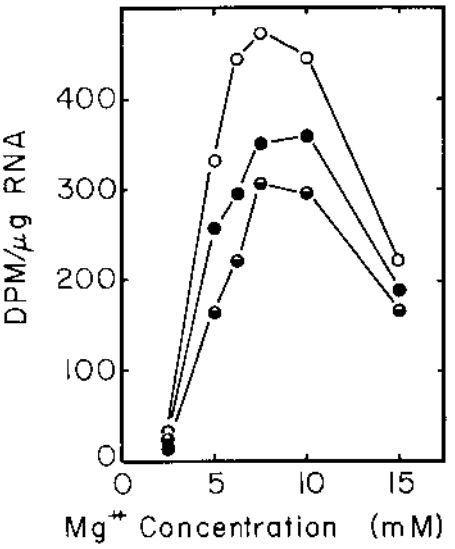

FIG. 2 -- Effect of Mg^{++} concentration on in vitro amino acid incorporation by the three regions of chick brain. The incorporation of radioactive valine into acid insoluble material was determined as described in the methods section for the cerebrum (○); cerebellum (●); and the optic lobes (◐) at the indicated concentrations of $MgCl_2$. Each point represents the average of three separate experiments.

activities. This difference was most apparent at the optimum Mg^{++} concentration. The in vitro amino acid incorporation by the cerebrum was 1.5 fold greater than the cerebellum or optic lobes. No significant difference was seen in the incorporation activities of the cerebellum and optic lobes. These differences will be seen in all following experiments reported herein.

Fig. 3 shows that the in vitro protein synthetic activity of chick brain was greatly affected by the K^+ concentration. For maximum activity all three regions of the chick brain required a K^+ concentration of 120 mM. Higher concentrations were inhibitory, especially to the cerebrum, until at a concentration of 225 mM, there was little difference in the in vitro protein synthetic activities of the three regions of the chick brain.

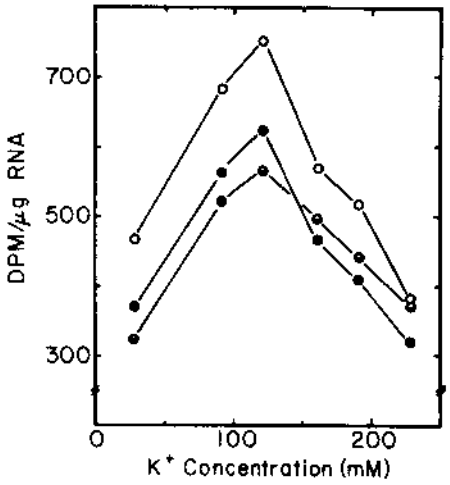

FIG. 3 -- Effect of K^+ concentration on the in vitro amino acid incorporation by chick brain. In all cases the pH of the final reaction mixture was 7.3 at 37°C, which was obtained by addition of KOH. The various levels of K^+ were obtained by varying the level of KCl in the reaction mixture. Incorporation of radioactive valine into acid insoluble material was determined as described in the methods section for the cerebrum (O); cerebellum (●); and optic lobes (●). Each point represents the average of three separate experiments.

It was of interest to determine what effect other monovalent cations had on in vitro protein synthesis by chick brain. Table 1 shows the effect of substitution of K^+ by ammonium (NH_4^+), sodium (Na^+), lithium (Li^+), and rubidium (Rb^+) ions. Concentrations of 50 mM or 93 mM of the different monovalent cations were added to reaction mixtures containing K^+ at a concentration of 70 mM or 27 mM respectively. With these combinations, the final concentration of monovalent cations was 120 mM. Comparing the amino acid incorporation obtained with 70 mM K^+ in the presence and absence of the various monovalent cations at 50 mM indicated that these ions could replace the K^+ requirement for in vitro protein synthesis by all three regions of the chick brain. When higher concentrations (93 mM) of the cations were added, neither Na^+ nor Li^+ could completely replace K^+. In fact the addition of 93 mM Na^+ was slightly inhibitory in the cerebrum and optic lobes when compared to control where no ion was added. Rb^+ and NH_4^+ ions at 93 mM were found to completely replace K^+. In the cerebrum substitution of 50 or 93 mM NH_4^+ stimulated protein synthesis 8-14%, while NH_4^+ had no stimulatory effect on amino acid incorporation by the cerebellum or optic lobes. Although the stimulation of in vitro amino acid incorporation in the cerebrum by NH_4^+ was slight, it was consistently observed at all concentrations tested. Similar studies with rat brain have shown that NH_4^+ ions can completely substitute for K^+ ions (Mahler, 1968) and increase amino acid incorporation 50% (Campagnoni, 1967). On the other hand substitution of K^+ ions by Na^+ ions has been shown to decrease in vitro protein synthesis by rat brain or rabbit cerebral cortex (Campagnoni, 1967; Zomzely-Neurath, 1972).

TABLE 1. THE EFFECT OF SODIUM, AMMONIUM, LITHIUM, AND RUBIDIUM IONS ON *IN VITRO* AMINO ACID INCORPORATION BY THE CHICK BRAIN

Ions added	Percent incorporation					
	cerebrum		cerebellum		optic lobes	
	50 mM	93 mM	50 mM	93 mM	50 mM	93 mM
No addition	81.9	62.4	86.6	60.8	85.8	65.6
K^+	100	100	100	100	100	100
Na^+	93.7	55.8	98.7	68.8	94.3	56.4
NH_4^+	113.7	108.2	104.3	100.4	95.3	94.2
Li^+	106.3	76.0	98.3	68.7	92.7	66.6
Rb^+	94.6	91.7	94.3	92.7	98.1	93.9

Fifty mM or 93 mM of the various monovalent cations was added to a reaction mixture containing 70 mM or 27 mM K^+. This results in a total concentration of monovalent cations of 120 mM (optimum concentration) except where no monovalent cation other than K^+ was added. In this case the concentration of monovalent cations was that of the K^+, 70 or 27 mM. The incorporation of radioactive valine into acid insoluble material was determined as described in the methods section. The incorporation was expressed as the percent of that obtained in the presence of 120 mM K^+. Each value represents the average of two separate experiments.

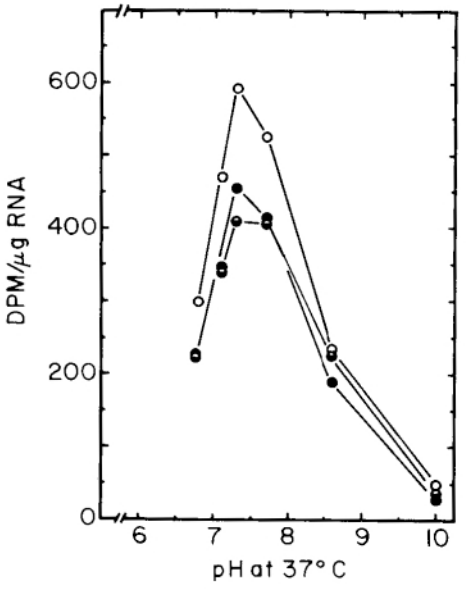

FIG. 4 -- Effect of pH on in vitro amino acid incorporation by the three regions of the chick brain. The homogenization buffer was maintained at pH 7.3, and the pH of incorporation medium was varied by adjusting the concentration of KCl and KOH of the incorporation medium so that final concentration of K^+ at all pH values was 120 mM. The pH values were determined by measuring the pH at 37°C of 1:1 mixtures of homogenization buffer and the incorporation media used for each reaction mixture. The incorporation of radioactive valine was determined as described in the methods section for the cerebrum (0); cerebellum (◒); and optic lobes (●). Each point represents the average of two separate experiments.

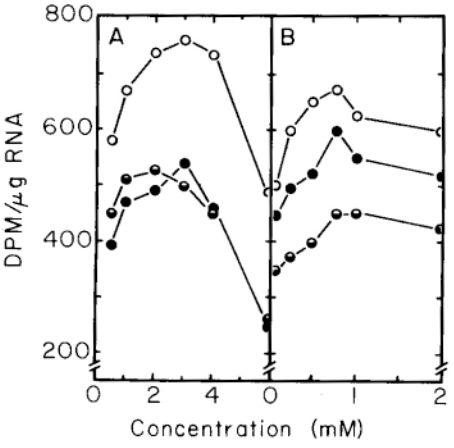

FIG. 5 -- Effect of ATP and GTP concentrations on in vitro amino acid incorporation by chick brain. Graph A shows the effect of various levels of ATP on amino acid incorporation at a constant GTP concentration of 0.75 mM. Graph B shows the effect of GTP concentration on amino acid incorporation at a constant ATP concentration of 3 mM. The incorporation of radioactive valine into acid insoluble material was determined as described in the methods section for the cerebrum (0); cerebellum (◒); and optic lobes (●). In both graphs, each point represents the average of three separate experiments.

The effect of the hydrogen ion concentration on in vitro amino acid incorporation by chick brain is shown in Fig. 4. The optimum pH was found to be 7.3 (at 37°C) for all three regions of the brain studied. Higher or lower pH values drastically reduced the level of amino acid incorporation obtained with each region of the chick brain. For the system described

herein, HEPES was the buffer used. The pKa of HEPES at 37°C has been shown by Good et al. (1966) to be 7.31 and, therefore, would be a very efficient buffer at pH 7.3. In addition, HEPES has been found to be superior to Tris as a buffering system for in vitro protein synthesis by rat liver (Hustor, 1970; Richardson, 1971).

ATP and GTP play essential roles in protein synthesis. GTP is the energy source for several reactions involved in the actual process of protein synthesis. On the other hand ATP serves as a source of energy in the formation of amino acyl tRNA and in the formation of GTP from GDP catalyzed by the enzyme nucleosidediphosphate kinase (EC 2.7.4.6) which would be present in the PMS. In the chick brain system creatine phosphate and creatine-phosphokinase were used as the energy-generating system. Omission of this energy-generating system resulted in a 45% decrease in amino acid incorporation. The creatine phosphate, creatine-phosphokinase system has been found superior to the phosphoenolpyruvate, pyruvate-kinase (EC 2.7.1.40) energy-generating system for in vitro protein synthesis in rabbit reticulocytes (Adamson, 1969) and rat liver (Falvey, 1970). Fig. 5 shows the effect of ATP and GTP concentrations on amino acid incorporation. The optimum concentrations of ATP and GTP appeared to be approximately 3 mM and 0.75 mM respectively for each region of the brain. In absence of ATP or GTP, incorporation was decreased by 80% and 50% respectively. Higher concentrations of ATP were quite inhibitory. This is probably due to the nucleotide triphosphate complexing with Mg^{++}.

It is obvious that an exogenous supply of L-amino acids would be required for in vitro protein synthesis to occur. However, it is generally quite difficult to demonstrate an amino acid requirement for in vitro protein synthesis (Richardson, 1971; Zomzely-Neurath, 1972). In order to obtain consistent results in this study it was necessary to wash all glassware very carefully to avoid even slight contamination of amino acids. Table 2 shows the effect of various amino acid levels on in vitro protein synthesis by chick brain. In the absence of amino acid supplementation incorporation was decreased 25-30%. The optimal level of amino acids appeared to be around 20 μM for all three regions of the chick brain. High concentrations of L-amino acids were observed to inhibit in vitro protein synthesis. Previous studies with rat liver (Liew, 1970; Wannemacher, 1970; Richardson, 1971) and rat brain (Folbergrova, 1966; Peterson, 1969; Lamar, 1971) have also shown that high levels of amino acids inhibit in vitro protein synthesis. Studies with rat brain indicate that this inhibition occurs after the formation of the pH 5 enzyme-AMP-amino acid complex but prior to the formation of the amino acyl tRNA molecule (Lamar, 1971).

DISCUSSION

Characterization of the first in vitro protein synthesis system from chick brain and factors affecting its activity have been described. Conditions for optimum in vitro protein synthesis by the cerebrum, cerebellum, and optic lobes of the chick brain were found to be identical. These conditions are similar, but not identical, to conditions previously reported for in vitro systems from rat brain (Campbell, 1966; Clouet, 1966; Zomzely-Neurath, 1972). The PMS system from chick brain described herein is very simple, can be obtained with few manipulations, requires only a small brain

Table 2 EFFECT OF AMINO ACID CONCENTRATION ON IN VITRO PROTEIN SYNTHESIS

Amino acid conc. (μM)	DPM per μg RNA		
	Cerebrum	Cerebellum	Optic lobes
0	636.2	456.3	548.4
0.5	653.0	508.1	560.1
2.5	728.2	560.6	631.8
7.5	860.3	612.4	689.7
20	888.9	641.6	747.1
50	877.5	583.3	728.5
200	753.8	535.0	606.8

The incorporation of radioactive valine into acid insoluble material was determined as described in methods except that the concentration of the L-amino acid mixture lacking L-valine was varied. The various concentrations of amino acids represents the concentration of each amino acid in the L-amino acid mixture. Each value represents the average of two separate experiments.

sample, and has a high protein synthetic activity. The amount of in vitro protein synthesis obtained from the chick brain cerebrum was 5-15 fold higher than those previously reported from rat brain (Campbell, 1966; Dunn, 1970; Murthy, 1972). The PMS from the cerebrum of 16-day-old embryos to 2-day-old chicks routinely incorporated 0.8 - 1.6 pmoles of L-valine per μg of RNA into acid insoluble material when incubated for 60 min at 37°C.

Two experiments suggest that in vitro initiation of protein synthesis is occurring in the chick brain system. First, kinetics of amino acid incorporation by this system are comparable to the kinetics obtained with a system from rabbit reticulocytes. Adamson et al. (1969) have shown that the addition of heme is necessary for in vitro initiation in the rabbit reticulocyte system. In the absence of heme the incorporation of radioactive amino acids was linear for 10 min at 37°C or 20 min at 26°C while amino acid incorporation was linear for 25 min at 37°C or 40 min at 26°C in the presence of heme (Adamson, 1968). In the chick brain system the incorporation of radioactive valine was linear for the first 10-20 min of incubation at 37°C and complete only after 40 min of incubation. At 25°C the incorporation of radioactive valine was linear for the first 30-35 min and not complete until after 60-70 min of incubation. Thus, the kinetics of amino acid incorporation by the PMS system from chick brain are comparable to the kinetics of

the rabbit reticulocyte system when heme is present and *in vitro* initiation of protein synthesis occurs.

One of the most interesting findings from this study was that different regions of the chick brain differ with respect to *in vitro* protein synthesis. For example, the presence of ammonium ions stimulate *in vitro* protein synthesis by the cerebrum 10-15% while no effect is apparent in the cerebellum or optic lobes. A higher rate of *in vitro* initiation by the PMS system from cerebrum might partially account for its higher protein synthetic activity. Studies are currently underway to determine why the *in vitro* protein synthetic activity of the three regions of the chick brain differ.

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