LASTING BIOLOGICAL EFFECTS OF EARLY ENVIRONMENTAL INFLUENCES

VII. Metabolism of Adenosine 3′,5′-Monophosphate in Mice Exposed to Early Environmental Stress

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As previously reported, we have studied the lasting effects of early infection or malnutrition on the physical development and metabolism of specific pathogen-free mice (1, 2). These stresses have been found to decrease the biosynthesis of protein and ribonucleic acid in various tissues and subcellular fractions of brain. Such metabolic derangements could be corrected by the combined administration of growth hormone and insulin (3). The possibility was considered that early environmental stresses caused abnormality in hormonal systems, thus producing secondary metabolic derangements in protein and ribonucleic acid metabolism. Changes in the intracellular levels of adenosine 3′,5′-monophosphate (cyclic AMP)[1] may be a generalized mechanism for the action of hormones in tissues (4, 5). Cyclic AMP has been shown furthermore to mediate the activation of brain protein kinase (6). Among mammalian tissues the brain has the highest activity of adenyl cyclase, the enzyme responsible for the synthesis of cyclic AMP (7), and the highest activity of cyclic 3′,5′-nucleotide phosphodiesterase, which inactivates cyclic AMP (8).

The present study was undertaken to investigate (*a*) the effects of neonatal infection or early malnutrition on the metabolic turnover of cyclic AMP in various organs and in synaptosomes in vitro, and (*b*) the regulatory mechanisms governing the brain level of cyclic AMP. As a measure of metabolic turnover we determined the catabolic activities in terms of production of respiratory $^{14}CO_2$ from cyclic AMP-8-^{14}C and its incorporation into various organs and into blood circulation. In addition, we determined the binding activities of synaptosomes for cyclic AMP. We also studied the changes of cyclic AMP caused by neonatal infection or by nutritional deprivation and the enzymatic activities regulating the brain level of nucleotides.

Materials and Methods

Experimental Animals and Enterovirus.—All experiments were carried out with specific pathogen-free mice of the COBS strain (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The origin and preparation of the mouse enterovirus have been described in an

[1] *Abbreviation used in this paper:* cyclic AMP, adenosine 3′, 5′-monophosphate.

earlier paper (9). The malnourished group consisted of the progeny of undernourished mothers fed a diet containing 20% wheat gluten as the sole protein source. The composition of the gluten diet is given in reference 3. The dams were placed on the gluten diet from day 14 of pregnancy to day 21 after delivery. After weaning all animals were transferred to D & G pellets (Dietrich and Gambrill Inc., Frederick, Md.), and kept on this complete diet *ad libitum*. Control animals were kept on D & G diet throughout the duration of the experiment.

Administration of Cyclic AMP-8-^{14}C and Measurement of Radioactivity in Respiratory Carbon Dioxide.—Experimental and control mice were fasted for 17 hr. Doses of 3.3 μCi/100 g body weight of cyclic AMP-8-^{14}C (Schwarz Bio Research Inc., Orangeburg, N.Y., specific activity 49.2 mCi/mmole, containing 6.6 μmoles/100 g body weight cyclic AMP) in a volume of about 0.2 ml was injected intraperitoneally. Immediately after injection, the animals were placed in 400-ml volume glass jars, which were tightly closed with rubber stoppers and connected with air inlet and outlet. The air flow was passed through a soda lime tube to remove carbon dioxide contamination and was maintained at a flow rate of about 1 liter/min. The respiratory carbon dioxide was collected at various intervals of time, 0–½, ½–1, 1–2, 2–3, 3–5, 5–7, 7–9 hr after injection into 10% phenethylamine solution in methanol, 8 ml/tube. Portions of sample solutions were mixed with a slight amount of solubilizer (Bio-Solv, Formula BBS-3, Beckman Instruments, Inc., Fullerton, Calif.) and 5 ml of scintillation fluid (10); the radioactivity was counted with a Packard scintillation spectrometer, model 3003 (Packard Instrument Co. Inc., Downers Grove, Ill.).

Incorporation of Cyclic AMP-8-^{14}C into Various Organs and Plasma.—The animals were killed 9 hr after injection. The liver, brain, and muscle were rapidly removed, weighed, and homogenized separately in ice-cold distilled water with a Teflon grinder. Portions of the homogenates were precipitated with 10% trichloroacetic acid in an ice bath for 10 min. The precipitates were separated by centrifugation and washed with 95% ethanol saturated with sodium acetate, then with ethanol:ether (3:1), and finally with ether to remove lipids. Portions of supernatants and of plasma samples were dissolved in solubilizer. The radioactivity of precipitates was measured by the combustion method (11). All samples were counted with a Packard scintillation spectrometer.

Chromatographic Separation of Metabolic Products of Cyclic AMP in Plasma.—Dowex 2 resin (AG2-X8, 100–200 mesh, chloride form, Bio-Rad Laboratories, Richmond, Calif.) was used for chromatography. The resin was washed with acetone a few times, then with an acetone-water solution (50%), followed by a final water wash. It was stirred with 2 N sodium hydroxide, filtered, and washed with distilled water until neutral, followed by washing with 2 N hydrochloric acid; the resin was then filtered and washed acid-free. Columns (1 × 40 cm) were packed with washed resin and 0.7 ml plasma samples were applied. The gradient elution system, based on the modified method of Nishizuka and Hayaishi (12), consisted of distilled water, 0.02 N, 0.1 N, 0.8 N, and 2 N formic acid, each in volume of 50 ml, and mixed by means of a nine-chambered gradient device (Buchler Instruments, Inc., Fort Lee, N. J.). After the gradient passed through, the column was eluted with 100 ml of 2 N formic acid. The optical density at 260 mμ in each 6 ml fraction was measured by spectrophotometry and the radioactivity in each fraction determined with a Packard scintillation spectrometer.

Preparation of Synaptosomes.—Synaptosomes were prepared by a modified method of cell fractionation (13). The fractionation scheme is shown in Fig. 1. Experimental and control mice were killed; cerebral cortices were removed rapidly and placed in cold 0.32 M sucrose. All operations were carried out at 0-4°C. A 10% (w/v) homogenate in 0.32 M sucrose was prepared in a Teflon homogenizer. The homogenate was centrifuged for 10 min at 1,000 *g* to separate the nuclei and unbroken tissue fragments (P_1). The supernatant (S_1) was centrifuged at 14,000 *g* for 15 min to yield a crude mitochondrial fraction (P_2) which was washed with 0.32 M sucrose and centrifuged at 15,000 *g* for 20 min (P_3). This crude mitochondrial pellet was resuspended in 0.32 M sucrose and layered on Ficoll gradients (Pharmacia Fine Chemicals, Inc., Uppsala,

Sweden). The gradients were prepared by placing 5.5 ml each of 20, 13, 10, 7.5, and 5% Ficoll in 0.32 M sucrose and equilibrated at 4°C for 30 min before use. The crude mitochondrial suspensions were centrifuged in SW 25.1 Spinco rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) at 25,000 *g* for 1 hr. The synaptosome fractions which layered between 7.5 and 13% were removed and combined. The synaptosome fractions were washed twice with

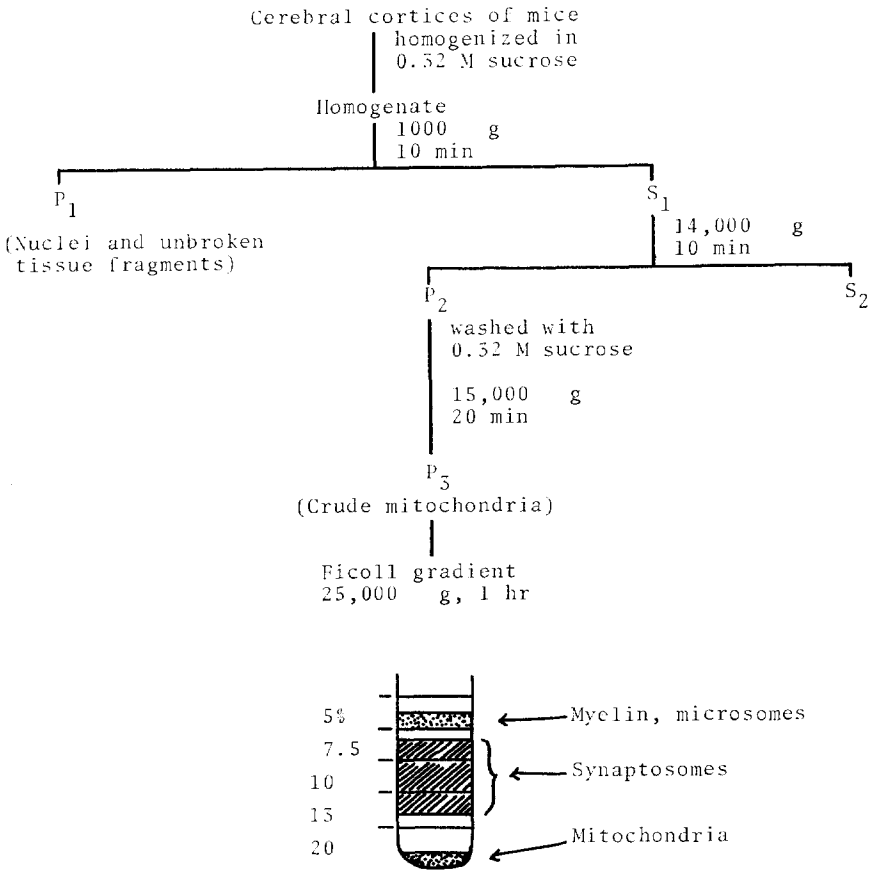

FIG. 1. Fractionation scheme used to obtain synaptosomal fraction of mouse brain.

0.32 M sucrose and centrifuged at 40,000 *g* for 30 min to remove Ficoll. The final pellet was suspended in 0.32 M sucrose solution.

Incorporation of Cyclic AMP-8-^{3}H into Synaptosomes.—Synaptosome aliquots containing 0.3 mg of protein were incubated in water bath at 37°C for 10, 20, 30, and 60 min. The incubation medium consisted of 0.01 M tris (hydroxymethyl) aminomethane (Tris)-HCl (pH 7.3), 2 mM magnesium chloride, 0.5 mM β-mercaptoethanol, and 1 μCi of cyclic AMP-8-^{3}H (specific activity 16.3 Ci/mmole, Schwarz Bio Research Inc.) in a final volume of 1.0 ml. Aliquots of incubation mixtures were precipitated with 10% trichloroacetic acid. In another aliquot, the the reactions were stopped by placing the incubation mixtures in ice. All samples were passed through a 0.45 μ Millipore filter and the insoluble residues were washed twice with cold incuba-

tion medium (without cyclic AMP-8-^{3}H). The radioactivity of residues was measured by the combustion method and counted with a Packard scintillation spectrometer, as described above.

The Binding Activity of Synaptosome for Cyclic AMP-8-^{3}H.—The reaction mixture consisted of 0.6 mg synaptosomal protein and a buffer solution, containing 0.01 M Tris-HCl (pH 7.3), 2 mM magnesium chloride, and 0.5 mM β-mercaptoethanol, in a final volume of 1.5 ml. The whole mixture was placed in a dialysis membrane tube (1 cm wide, Union Carbide Corporation, Chicago, Ill.) and dialyzed against 5 ml of 0.1 μCi cyclic AMP in the same buffer solution for 20 hr at 4°C on a rotary shaker. Equilibrium was attained under these conditions. The substrate bound to synaptosome was calculated by substracting the radioactivity of dialysate from that inside the tube.

Preparation of Sample Extracts for Determination of Cyclic AMP.—The sample extracts were prepared according to Breckenridge's method (14). Portions of brain homogenates were precipitated with equal volume of 10% trichloroacetic acid in an ice bath. After centrifugation, the supernatant fraction was separated and the trichloracetic acid removed with three portions of fresh water-saturated diethyl ether. Each portion of ether was equal in volume to 5–7 volumes of the supernatant fraction. The aqueous phase was heated 2 min in boiling water to expel residual ether.

Assay of Cyclic AMP.—The determination of cyclic AMP was based on the ability of this compound to accelerate the conversion of dephospho-phosphorylase to phosphorylase (15, 16). Phosphorylase activity was measured through its ability to catalyze the formation of inorganic phosphate from glucose-1-phosphate (17). Aliquots (0.1 ml) of a reagent containing 11.4 μmoles of Tris buffer (pH 7.4), 0.68 μmoles of magnesium sulfate, 0.54 μmoles of adenosine triphosphate (ATP), 0.27 μmoles of caffeine, and 0.03 μmole of ethylenediaminetetraacetate (EDTA) (pH 7.0) were pipetted into test tubes. Then to these were added 0.1 ml of 1.0 μmole Tris (pH 7.4) blanks, standard cyclic AMP solutions (in 1.0 μmole Tris, pH 7.4), or the sample extracts. Each tube received 0.1 ml mixture of 1.0 mg glycogen, 0.2 units of dephospho-phosphorylase (Worthington Biochemical Corporation, Freehold, N. J.), and 0.02 ml of an 11,000 *g* supernatant fraction of a 33% homogenate of mouse liver in 1.0 μmole Tris buffer (pH 7.4). The tubes were kept in an ice bath for exactly 30 min in order to increase the sensitivity of the assay. They were then transferred to a 25°C water bath for 20 min. During incubation, dephospho-phosphorylase was transformed to phosphorylase at a rate dependent on the concentration of cyclic AMP. The amount of phosphorylase present at the end of this incubation was determined by adding 0.1 ml 4% glycogen and 6.4 μmoles glucose-1-phosphate, the mixture being incubated at 37°C for 20 min. The reaction was terminated by adding 0.1 ml 5 N sulfuric acid. These mixtures were diluted to a volume of 4.8 ml with distilled water, to which were added 0.1 ml 2.5% ammonium molybdate and 0.1 ml reducing agent prepared according to Fiske and Subbarow (18). The mixtures were centrifuged and the absorbance of supernatant was read exactly 10 min later in a suitable photometer at 660 mμ. The absorbance of Tris blanks was subtracted from that of the cyclic AMP standards and the differences were plotted against the cyclic AMP concentration. In some cases the determination of cyclic AMP was reexamined by enzymatic assay (19). The effects of nucleotides, adenine, adenosine, glucose, or other substances which might interfere with the assay system of cyclic AMP have not yet been studied. It is assumed that such effects on the determination of cyclic AMP are negligible (20). Protein was determined colorimetrically (21).

Assay of Adenyl Cyclase.—Adenyl cyclase was assayed by the method of Sutherland et al. (7). Portions of brain homogenate were diluted in a 2 μmoles glycylglycine buffer, pH 7.4, and incubated in 1.0 ml of reaction mixture, containing 2 μmoles ATP, 3.5 μmoles magnesium sulfate, 6.7 μmoles caffeine, 10 μmoles sodium fluoride, and 40 μmoles Tris, pH 7.4, at 30°C for 15 min. At the end of incubation, the reaction was terminated by heating for 3 min in boiling water, followed by chilling. After centrifugation, the supernatant was assayed for cyclic AMP. Enzymatic activity was calculated from the net quantity of cyclic AMP produced during the incubation.

Assay of Phosphodiesterase.—5′-AMP produced from the cyclic AMP (catalyzed by the enzyme) was hydrolyzed by 5′-nucleotidase preparation (snake venom, *Crotalus atrox*, K & K Laboratories, Inc., Plainview, N.Y.) and the released inorganic phosphate was measured colorimetrically (22). The assay was carried out in a total volume of 0.9 ml containing 40

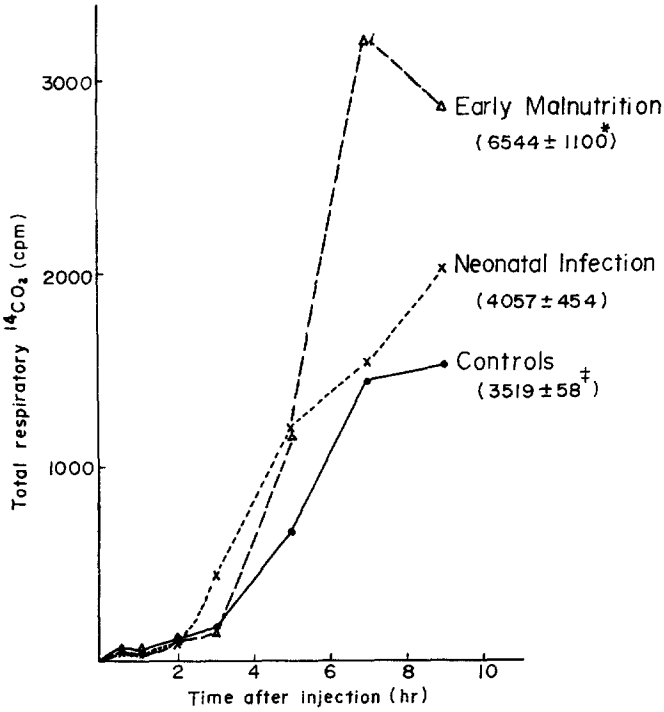

FIG. 2. Catabolic activity of cyclic AMP-8-^{14}C measured as conversion to respiratory $^{14}CO_2$. Altogether, five male mice per experimental group and five control males, 2 months old, received each 3.3 μCi/100 g body weight of cyclic AMP-8-^{14}C (containing 6.6 μmoles/100 g body weight cyclic AMP) by the intraperitoneal route. The samples of expired carbon dioxide were collected at indicated time intervals after injection. The parentheses indicate the total $^{14}CO_2$ expired in 9 hr.

*$P < 0.05$.

‡Mean of respective group ± SE.

μmoles Tris (pH 7.5), 1.0 μmole magnesium chloride, 1.0 μmole cyclic AMP, and sample preparation. The mixture was incubated at 30°C for 30 min. At 20 min, 0.1 mg *Crotalus atrox* snake venom in 0.1 ml of 0.1 μmole Tris (pH 7.5) was added and the mixture incubated for the remaining 10 min. The reaction was terminated by the addition of 0.1 ml cold 55% trichloroacetic acid; the liberated inorganic phosphate was determined colorimetrically (18).

RESULTS AND DISCUSSION

Metabolic Turnover of Cyclic AMP.—Fig. 2 shows the catabolism of cyclic AMP-8-^{14}C, expressed in conversion to respiratory $^{14}CO_2$, in mice having experi-

enced neonatal infection or malnutrition. The expiration of $^{14}CO_2$ from cyclic AMP-8-^{14}C was slow for 3 hr after injection; it increased rapidly from 3 to 7 hr and then gradually reached a plateau. The pattern of metabolic activity for cyclic AMP in terms of production of respiratory CO_2 was quite different from the utilization of acetate-^{14}C or glucose-^{14}C, which showed maximum expiration of $^{14}CO_2$ within a ½ hr after injection, then decreased rapidly in 5 hr (23). Cyclic AMP in experimental animals was also metabolized slowly during the 1st 2 hr. Then the production of respiratory CO_2 in the infected group increased

TABLE I

Incorporation of Cyclic AMP-8-^{14}C into Various Tissues and Plasma Level

Incorporation into tissues	Control	Neonatal infection	Early malnutrition
cpm/100 mg wet wt.			
Liver			
Supernatant fluid	26,575 ± 2438*	25,120 ± 1855	18,600 ± 2490‡
Sediment	1308 ± 385	341 ± 136‡	312 ± 67‡
Total	27,883 ± 2402	25,460 ± 1912	18,912 ± 2486‡
Brain			
Supernatant fluid	435 ± 29	224 ± 53‡	230 ± 66‡
Sediment	45.4 ± 3.7	33.8 ± 4.8	24.1 ± 5.2‡
Total	480 ± 28	257 ± 54‡	254 ± 69‡
Muscle			
Supernatant fluid	546 ± 38	364 ± 62‡	429 ± 40‡
Sediment	35.1 ± 2.9	32.7 ± 4.3	36.2 ± 4.6
Total	581 ± 41	397 ± 58‡	468 ± 34‡
Plasma level (cpm/ml)	5736 ± 893	3214 ± 453§	2959 ± 203§

Animals and experimental conditions were the same as in previous catabolic study of cyclic AMP-8-^{14}C (Fig. 2). Animals were killed at 9 hr after injection. Portions of tissue homogenates were precipitated with 10% trichloroacetic acid and the radioactivity of supernatant fluid and acid-precipitable fraction was measured.

* Mean of respective group ± SE.

‡ $P < 0.05$.

§ $P < 0.01$.

rapidly and continued to increase for at least 9 hr. In progeny of malnourished mothers, CO_2 production also increased rapidly; the expiration was much greater in these animals than in the controls for 5–7 hr after injection, then decreased. The total $^{14}CO_2$ expired in 9 hr was significantly higher than in the control group. The difference in total $^{14}CO_2$ expiration between infected and control groups was not statistically significant, perhaps due to the small number of animals and large variance, but the $^{14}CO_2$ expiration was still increasing in infected mice at the end of the 9 hr period.

Table I shows the incorporation of cyclic AMP-8-^{14}C into various tissues and into plasma. Most radioactivity (about 90–95% of total radioactivity) remained in the supernatant fluid of tissue homogenates. Only small amounts of cyclic

AMP-8-^{14}C were incorporated into the acid-precipitable fractions; it is possible that cyclic AMP was loosely bound to tissue macromolecules and could not be precipitated by trichloroacetic acid. Incorporation of cyclic AMP in liver was much higher than in brain or muscle. Total incorporation of cyclic AMP-8-^{14}C into almost all tissues studied was significantly decreased in the infected or malnourished groups. The incorporation into plasma was also lower in the experimental animals. These results suggest that the degradation of cyclic AMP to respiratory CO_2 was probably more active in the infected or malnourished groups; their plasma levels of cyclic AMP were lower, and the incorporations into various tissues were decreased.

Metabolic Products of Cyclic AMP in Plasma.—Fig. 3 shows the chromatographic separation of metabolic products of cyclic AMP in plasma. At 9 hr after injection of cyclic AMP-8-^{14}C, four peaks of radioactivity were observed: one peak corresponded to 5′-AMP, two peaks were located in the positions of adenosine and adenine, while one peak was not identified. This unknown peak did not correspond to adenosine diphosphate (ADP), hypoxanthine, xanthine, or certain inosine derivatives that could have developed during catabolism of cyclic AMP. Cyclic AMP-8-^{14}C disappeared totally from the plasma and may have been catabolized to 5′-AMP, adenosine, adenine, and other products. The major metabolites in the bile sample from the perfusion of rat liver with cyclic AMP-8-^{14}C have been reported to be 5′-AMP and guanosine (24). In the present experiment, however, guanosine did not seem to have been the catabolic product of cyclic AMP; instead, adenosine might be the major metabolite of cyclic AMP in mouse plasma. In infected and malnourished groups there were three major peaks of radioactivity; a peak corresponding to peak C in control group was not observed. Cyclic AMP-8-^{14}C also totally disappeared and more 5′-AMP accumulated in these animals. These results suggest that the metabolic activity from 5′-AMP to adenosine are slow in the experimental animals. The curve of optical density at 260 mμ did not match with the curve of radioactivity, particularly at the positions of 5′-AMP and cyclic AMP. The amount of 5′-AMP may have been too small to be detected by the optical density method, while in the position of cyclic AMP, the peak might be due to some nucleotides other than cyclic AMP. The control group showed five major peaks of optical density. A marked qualitative difference of optical density curve was found in the infected animals. The pattern of optical density curve in the malnourished group was similar to that in the control group.

Incorporation and Binding Activity of Cyclic AMP-8-^{3}H into Synaptosomes.—Fig. 4 shows the incorporation of cyclic AMP-8-^{3}H into total and precipitable fraction of synaptosomes. The synaptosomes were most active immediately after preparation and lost most of their activity after 24 hr at 4°C. The incorporation of cyclic AMP continued to increase up to 20 min then decreased rapidly in 60 min. Similarly to what had been observed in the in vivo cyclic AMP uptake into various tissues, small portions of cyclic AMP-8-^{3}H was

incorporated into the acid-precipitable fraction of synaptosomes. The ability to incorporate cyclic AMP into total or acid-precipitable fraction of synapto-

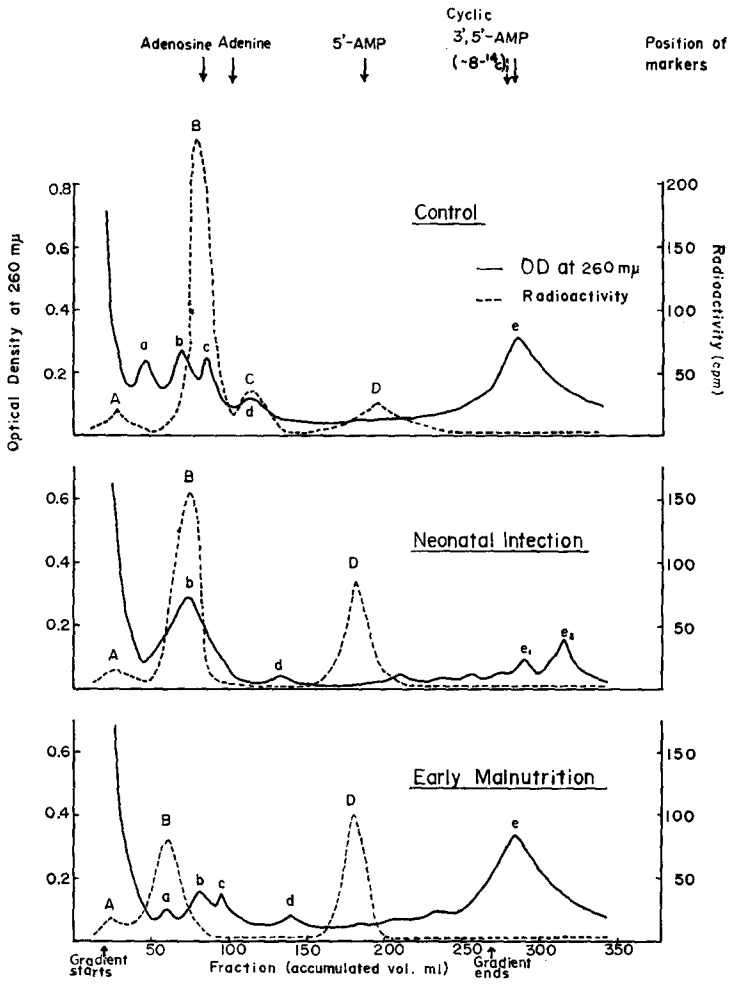

FIG. 3. Chromatographic separation of metabolic products of cyclic AMP in plasma on Dowex 2 formate column. Plasma samples were collected at 9 hr after intraperitoneal injection of cyclic AMP-8-^{14}C (3.3 μCi/100 g body weight). Positions of markers on chromatogram are indicated at the top of curves.

somes was lower in the infected and malnourished animals than in the controls.

Table II shows the effects of various physical and chemical treatments on the binding activity of synaptosomes for cyclic AMP. Neonatal infection or malnutrition decreased the binding activity of synaptosome for cyclic AMP, the activity being only 29–43% of that of the control group. The binding activity

was completely destroyed in all animals after treatment with trypsin, adjustment to pH 2 or 12, but not by treatment with cycloheximide, which can inhibit more than 70% of protein synthesis in synaptosome preparation, or by heating at 56°C. Addition of sodium and potassium ions appeared to inhibit

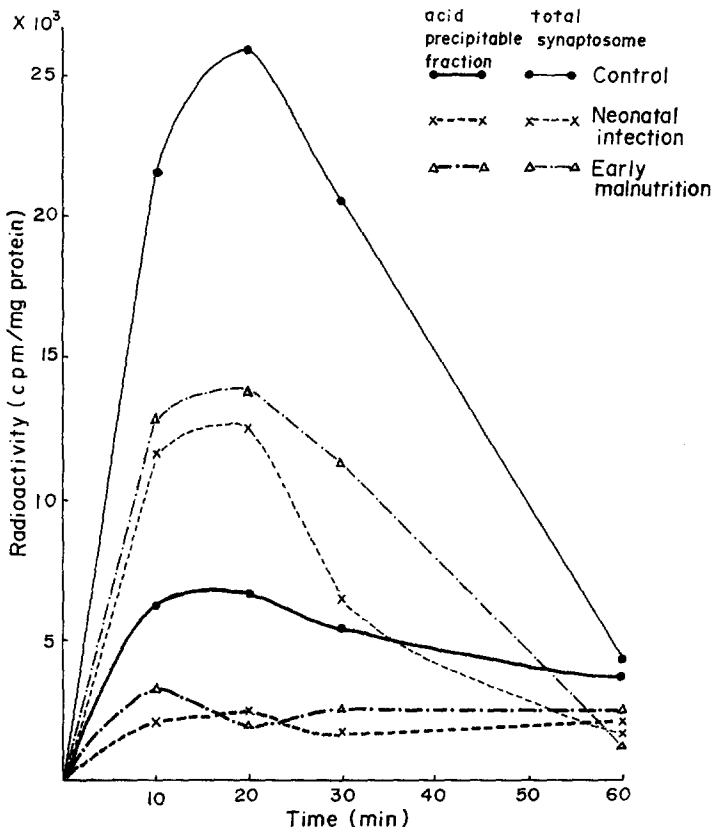

FIG. 4. Incorporation of cyclic AMP-8-^{3}H into synaptosomes. Reaction mixture was incubated at 37°C for the time indicated in 1.0 ml containing 0.01 M Tris-HCl (pH 7.3), 2 mM magnesium chloride, 0.5 mM β-mercaptoethanol, 1 μCi cyclic AMP-8-^{3}H, and 0.3 mg synaptosomal protein. The determination of radioactivity in total synaptosome and acid-precipitable fraction was processed as in Materials and Methods.

binding ability; in experimental animals, the activity decreased to 1–3% of the control group. In contrast, addition of EDTA, which binds ions and therefore removes their effect, greatly enhanced the binding activity of synaptosome. Addition of adenine nucleotides, such as AMP, or ATP also stimulated the binding activity. The incorporation of amino acid into synaptosomal protein has been reported to be relatively linear for 20 min and continued for approximately 90 min. Such incorporation is inhibited 70–80% by cycloheximide, is

not dependent upon exogenous substrates or energy supplies, including adenosine mono-, di-, and triphosphates, and is greatly stimulated by higher ionic contents of sodium and potassium (13). In the present study, the characteristics of incorporation of cyclic AMP into synaptosome are quite different from those of amino acid incorporation in that a decrease occurred after 20 min of incubation, perhaps due to the enzymatic degradation of cyclic AMP by phosphodiesterase activity. The incorporation of cyclic AMP was reduced to half by cycloheximide, greatly stimulated by AMP or ATP, and inhibited by

TABLE II

Effect of Various Physicochemical Treatments on the Binding Activity of Synaptosome for Cyclic AMP

	Control		Neonatal infection		Early malnutrition	
	cpm/mg protein	Relative activity	cpm/mg protein	Relative activity	cpm/mg protein	Relative activity
No treatment (pH 7.3)	1300	1.00	373	0.29	552	0.43
(picomoles/g protein)	(10.9)		(3.12)		(4.46)	
Treatment with trypsin (0.4%, 37°C for 1 hr)	No activity		No activity		No activity	
pH 2 adjustment	"	"	"	"	"	"
pH 12 "	"	"	"	"	"	"
+ $\begin{cases} Na^+ \text{ (2 mM)} \\ K^+ \text{ (0.2 mM)} \end{cases}$	362	0.28	18	0.01	33	0.03
+ EDTA (2 mM)	1540	1.18	2380	1.83	2720	2.09
+ Cycloheximide (1 mM)	617	0.48	182	0.14	382	0.29
Heating at 56°C	1240	0.96	850	0.65	930	0.72
+ 5'-AMP (1 mM)	2320	1.79	2380	1.83	2120	1.63
+ ATP (1 mM)	2380	1.83	2510	1.93	1350	1.04

The conditions for the determination of binding activity of synaptosome for cyclic AMP are described in Materials and Methods. The synaptosome was treated by various physical and chemical procedures. Comparative determinations of binding activity were made before and after each treatment.

higher ionic concentrations in the medium. Synaptosomes have been reported to contain relatively high concentrations of cyclic AMP (25), and may play some role in neurotransmitter storage and release, synaptic function, and mechanism of action of neurohormones (26–28). The decreased ability of infected and malnourished animals for the incorporation and binding activity of cyclic AMP into synaptosomes suggests the possibility of some impairment of cyclic AMP utilization and synaptic function in these animals.

Brain Cyclic AMP Concentration and Enzymatic Activities.—As seen in Table III, neonatal infection or malnutrition caused a significant decrease of brain cyclic AMP concentration, both in total value and on unit protein basis. The brain adenyl cyclase activity increased with age but was not affected in in-

fected or malnourished groups, while brain phosphodiesterase activity was elevated in these animals. A low concentration of brain cyclic AMP has been reported in anesthetized mice (29), perhaps due to the slow rate of metabolism and of conversion of inactive phosphorylase to active form. Phenothiazine derivatives also produced low cyclic AMP content, probably by inhibition of

TABLE III

Brain Cyclic AMP Content and Enzymatic Activities in Mice Exposed to Neonatal Infection or Malnutrition

	Control	Neonatal infection	Early malnutrition
Cyclic AMP content*			
Total (picomoles)	2.24 ± 0.09§	1.81 ± 0.07‖	1.88 ± 0.88¶
μmoles/100 g protein	8.77 ± 0.24	7.74 ± 0.25‖	7.44 ± 0.32‖
Enzymatic activities‡ (milliunit/mg protein)			
Adenyl cyclase			
2 months old	2.27 ± 0.12	2.21 ± 0.06	2.45 ± 0.31
4 " "	8.74 ± 0.55	9.78 ± 0.66	8.40 ± 0.62
Phosphodiesterase			
2 months old	2.58 ± 0.22	3.31 ± 0.18¶	3.32 ± 0.09¶
4 " "	4.48 ± 0.18	5.14 ± 0.15¶	5.35 ± 0.21‖

The preparation of sample extracts and conditions for the determination of cyclic AMP, adenyl cyclase, and phosphodiesterase are described in Materials and Methods. Enzyme assays for adenyl cyclase and phosphodiesterase were carried out on the same brain homogenate from each of control and experimental groups. One unit of adenyl cyclase was defined as that amount of enzyme which catalyzed the formation of 1.0 μmole of cyclic AMP in 15 min in the reaction mixture. One unit of phosphodiesterase was defined as the amount that caused the production of 1 μmole 5′-AMP from cyclic AMP/min at 30°C in reaction mixture.

* Altogether, 16 experimental (eight in each group) and eight control males, 4 wk of age, were used.

‡ Each group consisted of 16 males (eight at 2 months of age and eight at 4 months of age).

§ Mean of respective group ± SE.

‖ $P < 0.01$.

¶ $P < 0.05$.

adenyl cyclase activity of the pineal gland (30). An unidentified inhibitor of cyclic AMP was also found in some tissues (31).

The present study has revealed that neonatal infection or malnutrition experienced in early life have effects similar to that of anesthesia or certain chemical compounds, in that the level of brain cyclic AMP is lowered. Numerous agents have been shown to affect cyclic AMP accumulation, e.g., catecholamines, adrenocorticotropin (ACTH), or methylxanthine, etc. The effects of these substances are mediated through changes in the adenyl cyclase, which catalyzes the formation of cyclic AMP, or through an influence on phospho-

TABLE IV

Effect of Theophylline on Brain Phosphodiesterase Activity

Theophylline treatment	Enzyme activity		
	Control	Neonatal infection	Early malnutrition
	milliunit/mg protein		
−	1.45 ± 0.05*	1.94 ± 0.05	2.16 ± 0.15
+	1.37 ± 0.07	1.61 ± 0.08‡	1.50 ± 0.14‡

Each group consisted of eight males, 5 wk of age. Doses of 15 mg/kg body wt. theophylline, each in a volume of 0.1 ml were injected intraperitoneally. The animals were killed 1 hr after the injection. In groups not receiving theophylline, 0.9% sodium chloride solution was administered in the same volume and at the same time.

* Mean of respective group ± standard error of mean.

‡ $P < 0.01$. The statistical analysis was conducted in each group on the difference of results with and without the administration of theophylline.

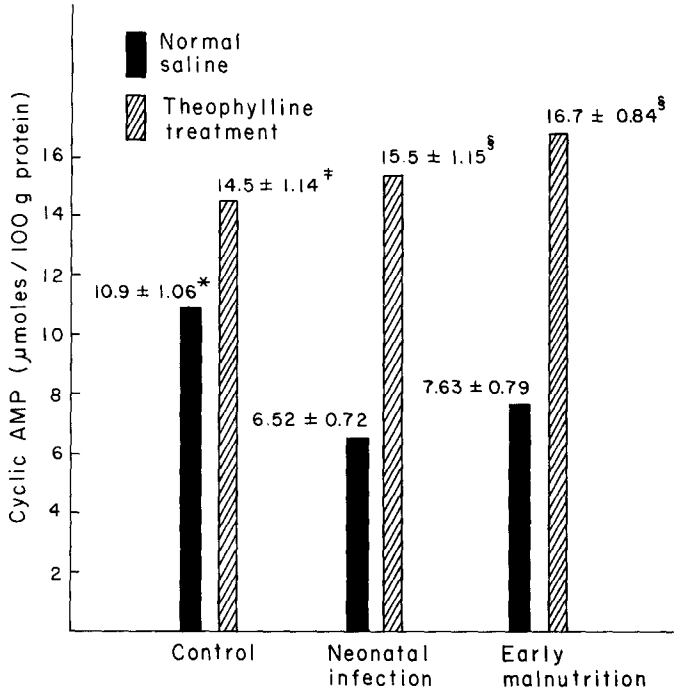

FIG. 5. Control and experimental groups used in the study were animals corresponding to those used in the previous experiment dealing with the response of brain phosphodiesterase activity to theophylline (Table IV).

*Mean of respective group ± SE.

‡$P < 0.05$.

§$P < 0.01$.

The statistical analysis was conducted in each group on the difference of results with and without the administration of theophylline.

diesterase, which inactivates cyclic AMP. Our experimental results suggest that the low concentration of brain cyclic AMP caused by neonatal infection or early malnutrition might result from the excessive activity of the enzyme which inactivates cyclic AMP, rather than from the impairment or lack of the enzyme synthesizing this compound.

Effect of Theophylline on the Accumulation of Brain Cyclic AMP.—Table IV shows the effect of theophylline on brain phosphodiesterase activity. This enzyme can be inhibited by methylxanthine derivatives, citrate, ATP, and pyrophosphate in vitro (32). As shown in an earlier experiment, its activity was significantly higher in infected or malnourished mice than in controls. The elevated activity of phosphodiesterase in experimental animals was brought back to normal by administration of theophylline.

As seen in Fig. 5 and in confirmation of earlier results, neonatal infection with enterovirus or malnutrition during early life resulted in a low concentration of brain cyclic AMP. The concentration of this compound was greatly increased in experimental as well as in control animals by administration of theophylline, perhaps as a secondary effect of phosphodiesterase response to the enzyme inhibitor.

SUMMARY

The metabolism of adenosine 3′,5′-monophosphate (cyclic AMP) was studied in specific pathogen-free mice exposed to neonatal infection with mouse enterovirus or to malnutrition during early life. Metabolic activity was determined by measuring the turnover of cyclic AMP-8-^{14}C to respiratory $^{14}CO_2$, its incorporation into various organs and plasma, and the binding activity of synaptosome for cyclic AMP.

Early malnutrition increased the catabolism of cyclic AMP as measured by expiration in respiratory CO_2. The level of cyclic AMP was lower in plasma and its incorporation into various tissues was decreased in infected and malnourished animals. Metabolic products of cyclic AMP were isolated from plasma by ion exchange chromatography. Cyclic AMP-8-^{14}C had completely disappeared 9 hr after injection. Fewer metabolites of cyclic AMP were detected in infected or malnourished groups than in controls and the metabolic reaction from 5′-AMP to adenosine seemed to be slow in these animals. The ability to incorporate cyclic AMP to synaptosome was also impaired in the experimental groups.

The concentrations of brain cyclic AMP were lower in infected or malnourished animals than in controls. Depression of accumulation of cyclic AMP probably resulted from excessive activity of phosphodiesterase, rather than from impairment of adenyl cyclase. Intraperitoneal administration of theophylline brought the activity level of phosphodiesterase to normal in infected or malnourished mice; this fact probably accounted for enhanced accumulation of brain cyclic AMP.

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