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Incorporation of L-Carbamyl-C^{14}-Aspartate into Acid-Soluble Pyrimidine Nucleotides of Mouse Brain.* (24663)

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Our previous studies(1) showed that propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in minced one-day-old mouse brain preparations results in increased uptake of P^{32} into ribonucleic acid phosphorus. Since it is known that acid-soluble nucleotides serve as precursors of cellular ribonucleic(2) and deoxyribonucleic acids(3), this study was undertaken to determine acid-soluble nucleotide constitution of noninfected and viral infected mouse brain and distribution of radioactivity in acid-soluble pyrimidine nucleotides and coenzymes following injection of a specific C^{14}-labeled pyrimidine nucleotide precursor, L-carbamyl-C^{14}-aspartate.

Methods. Synthesis of L-carbamyl-C^{14}-aspartate. L-carbamyl-C^{14}-aspartate was synthesized from urea-C^{14} (292 mg, specific activity 0.66 mc/mM), according to the method of Nyc and Mitchell(4). The alkaline reaction mixture was neutralized with concentrated HCl and the L-carbamyl-C^{14}-aspartate preparation was purified by separation on Dowex 1 (formate) X8 ion-exchange resin by the method of Lieberman and Kornberg (5). Based on the method of Koritz and Cohen(6) for determining carbamyl compounds, the specific activity of the final produce was 5.7×10^5 cpm/μmole. The calculated specific activity, based on urea, was 5.8×10^5 cpm/μmole. Although the L-carbamyl-C^{14}-aspartate solution was stored in deep-freeze, a chemical conversion to 5-carboxymethylhydantoin-C^{14} and to a smaller extent, dihydroorotate-C^{14} occurred. As much as 37% of total counts in the L-carbamyl-C^{14}-aspartate solution used for injection in Exp. 1 and 2 was present as 5-carboxymethylhydantoin. Chemical conversion was not detected when a neutralized L-carbamyl-C^{14}-aspartate solution was stored under similar conditions. *Injection of mice and preparation of acid-soluble fraction.* Mice, 5 to 6 weeks old, were injected intracranially in 6 sites with total of 0.024 ml L-carbamyl-C^{14}-aspartate solution (1×10^6 cpm/mouse). Mice were sacrificed 40 minutes after injection by cardiac exsanguination and brains were immediately dissected out and frozen by compression with dry ice. Frozen tissue was ground to a fine powder and homogenized with 2 volumes of ice-cold 0.6 N perchloric acid in tissue grinder, with a Teflon pestle. The homogenate was centrifuged and the protein pellet was extracted with 2 volumes of cold 0.2 N perchloric acid. Supernatant solutions from the 2 extractions were combined and perchloric acid was precipitated by addition of 5 N KOH to a phenol-red endpoint in an ice bath. After removal of $KClO_4$ by centrifugation, the perchloric acid-soluble fraction was chromatographed on a Dowex 1 (formate) X8 resin column. *Ion-exchange chromatography.* Purine and pyrimidine nucleotides present in perchloric acid-soluble extracts of mouse brain were separated by extended gradient elution chromatography system (modification of chromatographic system of Cohn(7) developed by Hurlbert *et al.* (8)). A more gradual formic acid gradient (mixer volume 1 liter, reservoir solution 2.3 N formic acid) was used for preliminary elution to get greater resolution of earlier

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TABLE I. Incorporation of L-Carbamyl-C^{14}-Aspartate into Acid-Soluble Pyrimidine Nucleotides of Mouse Brain after 40 Min. Exposure.

Nucleotide	Exp. 1* Total c.p.m.	Exp. 1* S.A.§	Exp. 2† Total c.p.m.	Exp. 2† S.A.	Exp. 3‡ Total c.p.m.	Exp. 3‡ S.A.
Carbamyl-aspartate	2×10^6		4.2×10^6		8.2×10^6	583,800
5-carboxymethyl-hydantoin	631,300		2.6×10^6		128,550	
Orotic acid	313,650	541,000	498,240	558,900	526,300	544,000
UMP	56,000	52,600	169,600	12,800	148,800	12,800
UDP	9,600	41,800	71,940	11,000	65,000	10,800
UTP			7,200	11,700	2,260	11,320
UDPG	2,600	26,000			7,200	9,450
UDPAG	4,750	12,000			10,750	4,460
CTP	560	6,000	330	1,500	240	1,300
CDP			580	1,170	360	1,100
CMP	680	2,400	1,360	930	3,210	900
CDP-ethanolamine					930	740

* 30 mice inj. with total 2.82×10^7 c.p.m. L-carbamyl-C^{14}-aspartate (S.A., 5.7×10^5).

† 100 mice inj. with total 1.17×10^8 c.p.m. Radioactive brain preparation diluted with extract 230 noninj. mouse brains.

‡ 90 mice inj. with total 9.08×10^7 c.p.m. Radioactive brain preparation diluted with extract 196 noninj. mouse brains.

§ Specific activity (S.A.) is count/min./μmole. Stand. dev. of counting rates were 2% or less.

peak components. *Analytical methods.* Isolated nucleotides were identified by absorption spectra in 0.01 N HCl, phosphorus analysis and in some instances by paper chromatography. Inorganic phosphate was determined by the method of Chen *et al.*(9). Total phosphate was determined after digestion with 6 meq sulfuric acid, labile phosphate following treatment of the sample with N H_2SO_4 for 15 minutes in a boiling water bath. Koritz and Cohen's(6) method for determination of carbamyl compounds was used for quantitative determination of carbamylaspartate and 5-carboxymethylhydantoin after quantitative conversion of the latter to carbamylaspartate by hydrolysis in N NaOH for 20 min at 100°. *C^{14} analysis.* Radioactivity of eluate samples was measured in 0.2 ml aliquots on aluminum planchets, 5.3 cm^2. Planchets were counted with an end-window counter (Model D-47, Nuclear-Chicago) and Nuclear Automatic Sample Changer. Radioactivity of purified nucleotide fractions was determined in duplicate, at infinite thinness with internal flow Q-gas counter (Model D-46, Nuclear-Chicago) on aluminum planchets.

Results. Nucleotide composition of adult Mouse Brain. The following purine and pyrimidine nucleotides were isolated and identified from mouse brain: AMP, ADP, ATP, GMP, GDP, CMP, CDP, CDP-ethanolamine, UMP, UDP, UTP, UDPAG and UDPG. In addition, a peak with A_{280}/A_{260} ratio corresponding to guanosine-containing compound and elution position characteristic of GTP was noted; this fraction was not further characterized.

Distribution of radioactivity. Distribution of radioactivity among perchloric acid-soluble components of mouse brain, 40 minutes after injection of L-carbamyl-C^{14}-aspartate into brains of 30 mice, is presented in Table I (Exp. 1). Several radioactive non-ultraviolet absorbing peaks were observed in addition to those of known pyrimidine nucleotides. Two of these were identified as 5-carboxymethylhydantoin and L-carbamylaspartate by carrier recrystallization and by ion-exchange chromatography following addition of nonradioactive material. Others were not identified. Specific activities of the recovered nucleotides are presented in Table I (Exp. 1). Specific activity of recovered orotic acid indicates little or no dilution by an endogenous pool. Labeling sequence of uridine nucleotides is similar to that in rat liver and in microorganisms(10). Specific activities of CMP and CTP are of particular interest since the higher level of C^{14}-incorporation into CTP indicates that uridine and cytidine nucleotide

interconversion in mouse brain occurs at the triphosphate level by a reaction similar to that in cell-free extracts of *E. coli*(11). Much radioactivity was found in 5-carboxymethylhydantoin, which was later found to be a contaminant of the injected L-carbamylaspartate solution.

To obtain sufficient material for characterization of unidentified radioactive peaks, of CDP and UTP, 100 mice were injected with L-carbamyl-C^{14}-aspartate and sacrificed 40 minutes after injection. Acid-soluble components were increased by addition of acid extract of 230 non-injected brains. Specific activities of recovered pyrimidine nucleotides are given in Table I (Exp. 2). Specific activities of recovered pyrimidine compounds were about one-third those in Exp. 1, except for orotic acid. Since the specific activity of orotic acid was not changed by dilution with extract of noninjected brains, endogenous orotic acid level of mouse brain probably is very small. The results verify the conclusions from Exp. 1. Although more counts were recovered in radioactive nonultraviolet absorbing fractions than in the previous experiment, the amounts were insufficient for characterization.

To test the effect of 5-carboxymethylhydantoin-C^{14} on conversion of L-carbamyl-C^{14}-aspartate to pyrimidine nucleotides, 90 mice were injected with a total of 9.08×10^7 cpm of carbamylaspartate solution containing only 1% of the total radioactivity as contaminating 5-carboxymethylhydantoin. Animals were sacrificed 40 minutes after injection of isotopic material. Extract of radioactive brains was diluted with extract from brains of 196 noninjured animals. After injection of purified L-carbamyl-C^{14}-aspartate solution, all non-ultraviolet absorbing radioactive fractions except 5-carboxymethylhydantoin and carbamylaspartate were absent. Specific activities of pyrimidine nucleotides in this experiment (Table I, Exp. 3) are in good agreement with those from animals injected with mixture of 5-carboxymethylhydantoin and L-carbamylaspartate. This suggests that injected radioactive 5-carboxymethylhydantoin does not significantly affect conversion of carbamylaspartate to pyrimidine nucleotides. 5-carboxymethylhydantoin-C^{14} and L-carbamyl-C^{14} were recovered from acid-solubles in about same ratio as was present in the injected solution. This suggests that there was little or no conversion of L-carbamyl-C^{14}-aspartate to 5-carboxymethylhydantoin, *in vivo*.

Ability of mouse brain tissue to carry out the reverse reaction, conversion of 5-carboxymethylhydantoin to carbamylaspartate, was tested by injection of 36 mice with a total of 2.28×10^7 cpm purified 5-carboxymethylhydantoin-C^{14}. Animals were sacrificed 40 minutes later. Six radioactive fractions were recovered from perchloric acid-soluble fraction. Three of these were identified respectively as 5-carboxymethylhydantoin (2.4×10^6 cpm), carbamylaspartate (14,000 cpm) and UMP (1000 cpm). Remaining fractions possessed elution characteristics of non-ultraviolet absorbing radioactive peaks observed in Exp. 1 and 2. These results show that metabolic conversion of 5-carboxymethylhydantoin to carbamylaspartate does not occur to any great extent in mouse brain.

Virus infected mouse brain. Distribution of C^{14} in acid-soluble nucleotides of mouse brain was determined after injection of L-carbamyl-C^{14}-aspartate into mice previously infected with Theiler's GD VII strain of mouse encephalomyelitis virus. Forty-three mice were each injected intracerebrally with 0.03 ml virus (10^3 MLD_{50}). Virus-infected mice were injected intracranially with L-carbamyl-C^{14}-aspartate after first paralytic signs in the group, about 72 hours after injection of virus. Thirty-six mice were sacrificed 40 minutes later. Neutralized perchloric acid-soluble extract was made and chromatographed. Resultant chromatogram was almost identical with that for noninfected brain tissue. No significant differences were found in quantities of ultraviolet absorbing and radioactive materials of virus-infected and normal mouse brain. Specific activities of 3 radioactive pyrimidine nucleotides from virus-infected preparation, UMP-CMP and CDP were identical with those in noninfected brain.

Discussion. The acid-soluble nucleotide composition of mouse brain tissue is similar to that of other tissues(8,12,13). Specific

activities of isolated pyrimidine nucleotides labeled from L-carbamyl-C^{14}-aspartate indicate that pyrimidine biosynthesis in mouse brain proceeds through a sequence of reactions which have been shown to occur in other tissues(10). Specific activity of recovered orotic acid suggests that there was very little dilution of the radioactivity of carbamylaspartate by unlabeled metabolic pools of dihydroorotate and orotate. Hurlbert and Potter(14) obtained evidence that the size of the metabolic pool of orotic acid in rat liver is very small. When the distribution of radioactivity of rat liver nucleotides was determined, following administration of orotate-C^{14}, practically all radioactivity could be accounted for as uridine nucleotides and no radioactive orotate, dihydroorotate or orotidylate were recovered. Thus it is probable that dihydroorotate and orotidylate which were not detected in the acid-soluble fraction from mouse brain, are present in very small amounts as intermediates of transient existence in mouse brain tissue. This assumption would account for the finding that carbamylaspartate is converted to orotate without dilution.

Incorporation of carbon-14 into all uridine nucleotides was more rapid than into cytidine nucleotides, thus fulfilling one criterion for a precursor-product relationship. The specific activity values of the uridine and cytidine nucleotides are in accord with the fact that the only known reaction for conversion of a uridine to a cytidine compound is that shown to occur at the nucleoside triphosphate level by Lieberman(11) with an extract of *E. coli*.

Ion-exchange chromatography of acid-soluble nucleotide fraction of mouse brains injected with stored solution of L-carbamyl-C^{14}-aspartate solution revealed presence of several radioactive non-ultraviolet absorbing peaks in addition to carbamylaspartate and 5-carboxymethylhydantoin. These unidentified compounds were not detected when pure L-carbamyl-C^{14}-aspartate was injected into the mice but did appear in each instance when 5-carboxymethylhydantoin-C^{14} was administered. This would imply that they are not normal intermediates of carbamylaspartate metabolism but may represent catabolic products of 5-carboxymethylhydantoin. The data also show little conversion of 5-carboxymethylhydantoin to carbamylaspartate; hence the former compound is not readily converted to pyrimidine nucleotide compounds.

From previous studies in this laboratory which showed a stimulatory effect of propagation of Theiler's GD VII virus on uptake of P^{32} into RNA of minced one-day-old mouse brain preparations(1), it is felt that the apparent similarity in distribution of the label of L-carbamyl-C^{14}-aspartate among the acid-soluble pyrimidine nucleotides of virus-infected and non-infected tissues does not reflect a lack of effect of virus propagation on acid-soluble pyrimidine metabolism of the tissue, *in vivo*, but rather serves to emphasize the need for a cellular system in which homogeneity of the cell population and infection of most of the cells by the virus can be more adequately controlled. Work is now in progress to develop such a system with mouse brain tumor tissue culture cells.

Summary. Acid-soluble pyrimidine nucleotides were separated by gradient elution ion-exchange chromatography after intracranial injection of L-carbamyl-C^{14}-aspartate into mice. Radioactivity was associated with orotic acid, UMP, UDP, UTP, UDPAG, UDPG, CTP, CDP, CMP and CDP-ethanolamine; this shows that brain tissue is capable of synthesizing these pyrimidine nucleotides from simple precursors. Specific activities of isolated pyrimidine nucleotides indicate that biosynthesis of pyrimidine nucleotides by brain tissue occurs in a manner similar to that in rat liver and bacterial extracts. Comparison of specific activities of CTP, CDP and CMP suggests that uridine to cytidine nucleotide conversion takes place between UTP and CTP in mouse brain, probably by a reaction similar to that shown by others in extracts of *E. coli*. Distribution of radioactivity was the same in brains of mice previously infected with Theiler's encephalomyelitis virus and in noninfected brains.

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Transfer of Experimental Panophthalmitis in Rats by Parabiosis.*† (24664)

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Transfer of experimental encephalomyelitis and glomerulonephritis by parabiosis was accomplished in rats(1,2). Transfer of glomerulonephritis has been demonstrated in both random bred and inbred strains of rats (3-5). The question arose, however, whether these transfer phenomena also held true for sensitization processes in other organs. In our experiments, allergic panophthalmitis has been produced by injecting rabbit-anti-rat-eye-serum into rats. The ophthalmitic animals were then joined with normal rats in parabiosis. Here, too, transfer of inflammation of the eye on the originally healthy partners has been achieved.

Methods. a) *Rabbit-anti-rat-eye-serum.* Frozen eyeballs of white rats of different breeds were triturated by the ballmill, diluted with saline to 20% suspension and injected 3 times a week for 2 months, and subsequently twice a week during 1 month, intraperitoneally into rabbits weighing 3-4 kg. Then the rabbits were bled from carotid arteries, blood collected under aseptic conditions, and antisera pooled and stored in 10 ml amounts in deep-freezer. b). *Cytotoxic panophthalmitis.* White rats of one strain (Stamm Zeilsheim) weighing 80-100 g, were injected with cytotoxic rabbit-anti-rat-eye-serum. Most animals were injected intravenously with single dose of 1-1, 8 ml of antiserum. Some rats also received that amount in 2 to 5 more daily injections. Starting 24 hours after first injection, the fundi of eyes were examined ophthalmoscopically every second day during experiment. The rats were sacrificed between 5th and 86th day after application of the antiserum. Whole eyes were fixed in 4% formalin, and sections of whole eye as well as of different tissues of eye were stained with H. + E. and PAS for microscopic examination. Sections of brain, heart, liver, spleen, and kidneys also were fixed in 4% formalin and stained with H. + E. Rats of the same strain, serving as controls, were untreated or injected with normal rabbit serum. Others were injected with 1 ml rabbit-anti-rat-kidney-serum intravenously. c) *Parabiosis.* As in former experiments(2) the parabiotic opera-

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