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Incorporation of Adenine-8-C-14 and Orotic-6-C-14 Acid into Nucleic Acids of the Feline Neuraxis.* (23708)

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Present knowledge of nucleic acid metabolism in the mammalian neural axis is derived principally from histological studies and from the application of ultraviolet microspectroscopy to tissue sections(1-3). The glial elements have been generally neglected, nerve cell bodies being the chief object of study. Turnover studies involving orthophosphate-P-32(4-6) have revealed incorporation into nucleic acid phosphate in whole mammalian brain, but, according to Strickland(7), the values reported earlier are high because of contamination of the nucleic acid fraction by other phosphate-containing compounds of high specific activity. This author isolated the nucleic acids from the brains of cats given phosphate-P-32 intracisternally and separated them into pentose nucleic acid (PNA) and deoxypentose nucleic acid (DNA) by alkaline hydrolysis. He observed appreciable incorporation of P-32 into PNA, but only slight incorporation into DNA. In subsequent papers from Rossiter's laboratory(8,9), it was demonstrated that an oxidative phosphorylating mechanism was involved in the incorporation of phosphate-P-32 into the PNA of cat brain slices *in vitro*. Bendich *et al.*(10) reported slight incorporation of formate-C-14 into brain PNA as compared with other viscera. However, they did not consider the question of the availability of the labeled precursor to the brain, *i.e.*, the blood-brain barrier. This communication presents the biochemical results obtained with the use of a labeled purine and a labeled pyrimidine precursor administered intrathecally in a series of adult cats. Avoidance of the blood-brain barrier by injection of the

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precursor directly into the cerebrospinal fluid permitted attainment of adequate levels of radioactivity with minimal doses of these expensive chemicals.

Methods. Adenine-8-C-14 (15.4×10^6 counts/min./mg) and orotic-6-C-14 acid (7.3×10^6 counts/min./mg), purine and pyrimidine precursors respectively, were injected intracisternally and in some instances into the lumbar subarachnoid space following laminectomy in cats anesthetized with sodium pentobarbital. Animals were killed under anesthesia by exsanguination at various time intervals following injection. The brain and cervical spinal cord with attached nerve roots and ganglia (also the lumbosacral spinal cord and radicular structures when intraspinal injection was performed) were removed and after appropriate tissue blocks were removed and placed in neutral 10% formalin for autoradiography, the remainder of the neural axis was preserved in a deep freeze at $-10°$ to $-20°$ until the biochemical studies could be carried out. The autoradiographic study is being reported in a separate communication. The spinal cord segments were maintained in the frozen state on ice while they were carefully dissected into gray and white matter for separate analysis; in some experiments spinal nerve roots and ganglia were also separated for analysis. A portion of the hindbrain was employed without further dissection. The tissue samples were weighed on a torsion balance and homogenized in 5 volumes of 10% NaCl (1 ml was the minimum volume used although samples of the ganglia and nerve roots frequently weighed less than 100 mg). The nucleic acids were extracted by boiling the homogenate in a water bath for one hour, removing the supernatant solution and boiling the residue in 2½ volume of 10% NaCl for ½ to 1 hour longer.† The combined NaCl solutions were chilled and mixed with 3 volumes of cold 95% ethanol. The precipitated sodium nucleates were removed by centrifugation, washed with cold 95% ethanol and with .05 N HCl, dried and incubated in 1-2 ml of .1N NaOH for 18-20 hours at 37°C. The DNA was precipitated by making the alkaline digest .1N acid with HCl, the PNA remaining in the supernatant solution in the form of its constituent mononucleotides. The DNA precipitate was thoroughly washed in .05N HCl, dissolved in .1N NaOH, reprecipitated with 1N HCl to remove contaminating traces of ribonucleotides and dissolved in .02N NaOH. Further washing and reprecipitation did not alter the specific activity of DNA.‡ Aliquots of the separated nucleic acid solutions were plated in duplicate on aluminum planchets for measurement of radioactivity; an aliquot was delivered from the same pipette into a test tube, and after dilution the absorbance at 260 mμ was measured in a Beckman spectrophotometer in order to estimate the amount of nucleic acid plated.§ The amount of PNA plated for counting

† Many authors have employed 10% NaCl for extraction of nucleic acids from tissues and alkaline hydrolysis for separation of PNA and DNA. Our procedure is the same as that of Hurlbert and Potter (11) with the exception that fresh frozen tissues were routinely used instead of acid- and lipid-extracted tissues. Control experiments revealed that omission of preliminary extraction in cold acid and in hot ethanol-ether did not alter the specific activity of isolated nucleic acids and that the specific activity remained constant in nucleic acids derived from successive extractions of tissues in boiling salt solution.

‡ Analysis of several samples of brain DNA for pentose revealed the presence of 2-3.5% PNA. This is in agreement with Hurlbert and Potter(11) who found their DNA fraction contained less than 5% PNA. Histochemical studies(15) employing fixed specimens from tissues analyzed in our report revealed that most of the residual radioactivity was extractable from tissue sections by ribonuclease. In one experiment (CA5), sections of formalin-fixed spinal cord and brain which had been subjected to digestion by ribonuclease until no further radioactivity could be extracted lost a significant amount of radioactivity upon incubation in crystalline deoxyribonuclease solution. These experiments provide qualitative evidence for the presence of radioactivity in PNA and DNA.

§ The amounts of PNA and DNA obtained depended upon the concentration of nucleic acids in the neural tissues studied and size of available samples. From 100-750 μg of each nucleic acid was extracted from spinal white and gray matter, 10-100 μg from spinal ganglia and roots and 1000-5000 μg from hindbrain.

TABLE I. Incorporation of Adenine-8-C-14 into Nucleic Acids of Neural Axis of the Mature Cat.

				S.A.* PNA/DNA				
				Cervical spinal cord				
Exp.	Dose & route† (× 10^6 cpm)	Time interval	White matter	Gray matter	Spinal ganglia	Sp. nerve roots	Hindbrain‡
CA 9	30.7 IC	1 hr	14,650	3,260			1,270
	30.7 IS		775	760			54
10	15.4 IC	2	61,000	3,520	1,700	21,200	5,720
			433	117	230	820	237
8	15.4 "	4	12,500	3,380	2,440		418
	15.4 IS		593	945	960		23
11	15.4 IC	24	81,000	39,200	10,600	88,400§	38,000
			620	506	322	513§	261
12	15.4 "	46	21,600	3,610	3,270	56,300§	3,550
			640	245	455	480§	51
3	15.4 "	9 days	77,800	64,500			9,950
			2,330	2,250			285
7	20.5 "	51	30,800	23,000	3,050		30,000
			24,500	12,800	2,660		4,730
			53,500‖	15,700‖	3,840‖	19,900‖	
			38,200‖	14,900‖	2,250‖	17,000‖	
5	69 "	9	236,000	174,000			66,800
	in 5 daily inj.		48,300	31,500			2,020

* Specific activity (counts/min./mg nucleic acid). Nucleic acid was calculated from the absorbance at 260 mμ.

† IC = intracisternal inj. IS = intraspinal inj. S.A. of adenine-8-C-14 = 15.4 × 10^6 cpm per mg.

‡ Medulla, pons and cerebellum.

§ Accuracy questionable because of small sample size.

‖ Lumbosacral spinal cord.

was calculated from the formula $\mu g\ PNA = \frac{A260 \times V}{.0318}$ where A260 = absorbance at 260 mμ (1 cm path), V = volume to which aliquot is diluted and .0318 is the absorbance at 260 mμ of a hydrolyzed acidified solution of yeast PNA (N = 15.5%, P = 8.9%) containing 1 μg/ml. The amount of DNA plated for counting was calculated from the formula $\mu g\ DNA = \frac{A260}{.019} \times V$ where .019 is the absorbance at 260 mμ of a solution of purified calf thymus DNA (N = 15.3%, P = 9.1%) in .005 N NaOH containing one μg/ml. All samples were counted in a gas flow counter (Nuclear Model D 47 equipped with "micromil" end window) in conjunction with an automatic sample changer to give a standard error in counting of 3% or less; appropriate corrections for background, self-absorption of sample and counter efficiency were made in calculating the specific activity (S.A.) of PNA and DNA.

Results. The results are presented in Tables I and II. It is evident that labeled adenine and orotic acid were rapidly incorporated into PNA in the central nervous system of the adult cat. The specific activity (S.A.) varied considerably from experiment to experiment; this may be attributable to unavoidable differences in local concentration of labeled compounds which may be affected by factors such as speed of injection, cerebrospinal fluid movement, diffusion away into the blood stream and others. However, the retention of labeled precursors, particularly adenine, in the nucleic acids of the neural axis for as long as 51 days is striking. In general, the DNA fraction was labeled only slightly as compared with PNA and could be caused by labeled contaminants, particularly PNA.‡ In animals receiving labeled precursors in

TABLE II. Incorporation of Orotic-6-C-14 Acid into Nucleic Acids of Neural Axis of the Mature Cat.

			S.A.* PNA / DNA				
			Cervical spinal cord				
Exp.	Dose & route† ($\times 10^6$ cpm)	Time interval	White matter	Gray matter	Spinal ganglia	Sp. nerve roots	Hindbrain‡
CO 6	7.3 IC	4.5 hr	11,350	14,700			6,550
			780	495			89
4	14.5 "	4	6,550	5,080	805		16,200
	" IS		98	133	184		151
7	" IC	23	90,500	57,000	4,800	107,000	37,200
			375	485	485	400	245
2	7.3 "	43	5,460	447	758§	15,300§	930
			143	154	506§	116§	5
3	21.8 "	44	58,200	72,000			16,000
			327	306			31
5	14.5 "	51 days	5,250	5,670			6,400
	" IS		2,510	1,260			1,960
			1,980	1,420‖	298‖	1,950‖	
			1,010	1,310‖	300‖	1,620‖	
8	58.1 IC	15	105,500	118,500	5,870	82,300	85,000
	in 4 daily inj.		11,850	2,620	371	2,870	1,780

* Specific activity (counts/min./mg nucleic acid). Nucleic acid was calculated from the absorbance at 260 mμ.

† IC = intracisternal inj. IS = intraspinal inj. S.A. of orotic acid-6-C-14 = 7.3×10^6 cpm per mg.

‡ Medulla, pons and cerebellum.

§ Accuracy questionable because of small sample size.

‖ Lumbosacral spinal cord.

relatively large doses by multiple daily injections and in animals surviving for 51 days following a single injection, the labeling of DNA is probably significant; in these experiments the specific activity of the DNA fraction approached the specific activity of PNA in many of the tissue samples (experiments CA7, CA5, CO5 and CO8). However, while these data suggest incorporation of labeled precursors into DNA under the conditions of these experiments, isolation and measurement of specific activity of constituent mononucleotides is required to prove such incorporation. It is particularly important to substantiate these findings because cell division probably does not occur to any significant degree in the central nervous system of healthy adult mammals(12) and DNA is widely believed to be metabolically stable in the absence of cell division(13). If this should prove to be true incorporation into DNA, it would follow that the labeled precursor was derived either directly or indirectly from that previously incorporated into PNA. Work is underway to resolve this question.

The PNA of white matter of spinal cord exhibited consistently high S.A.; with adenine it always exceeded the S.A. of PNA derived from gray matter of the same segments. In some experiments the precursors appear to have been metabolized and fixed so rapidly as it diffused through the outer white matter that relatively little penetrated into the centrally placed spinal gray during the elapsed time. The S.A. of PNA from spinal nerve roots was also high; in sharp contrast to this, the S.A. of PNA of spinal ganglia was relatively low. The PNA of hindbrain was generally less active than that of cervical spinal cord. This is believed to be a reflection of local differences in movement of cerebrospinal fluid and diffusion distance in neural tissue rather than an intrinsic difference in nucleic acid incorporation in these two parts of the neural axis. In those experiments in which DNA may have been significantly labeled, *i.e.*,

long term and multiple injection experiments, *vide supra*, the distributional pattern followed closely that of PNA. The DNA fraction of spinal white was generally most active, that of spinal gray and spinal nerve roots somewhat less active and that of spinal ganglia least active.

The extremely rapid incorporation of labeled precursors into PNA and the prolonged retention of these precursors in PNA in the neural axis appear incongruous at first sight. However, these findings can be reconciled if it is assumed that the catabolic products are used over and over in the dynamic turnover of PNA. Indeed, the presence of a blood-brain barrier which greatly restricts penetration of nucleic acid precursors would make such conservation useful if not essential. The high turnover of PNA in spinal white matter indicates the occurrence of active nucleic acid metabolism in glial cells. This conclusion is supported by results of autoradiographic studies carried out in this laboratory(13), which reveal labeling of PNA of interfascicular oligodendrocytes in the white matter of spinal cord and brain. The same conclusion appears warranted for the glial and Schwann cells of the spinal nerve roots. Incorporation into PNA of glial cells and nerve cells in gray matter has been observed in autoradiographs (14). It is interesting to note that nuclear PNA becomes labeled some hours before cytoplasmic PNA in neurons within the central nervous system. Spinal ganglion neurons and surrounding satellite cells manifest a much less active incorporation of adenine or orotic acid into their PNA in autoradiographs. This agrees with the biochemical results and appears to be a significant metabolic difference between the multipolar neurons within the neuraxis and the unipolar spinal ganglion neurons.

There is much evidence of an association between PNA and protein synthesis; cells rich in PNA also actively synthesize protein(16). However, the form of this association is not yet understood. The abundance of PNA in neurons has been related to protein synthesis by Hydén(1,3). Histochemical and autoradiographic studies by others(17-20) have demonstrated active incorporation of this labeled amino acid into nerve cell protein. Weiss and Hiscoe's demonstration(21) of a centrifugal flow of axoplasm in peripheral nerves of adult mammals suggests a purpose for protein synthesis by the neuron soma, namely, replacement of axoplasm dissipated in the peripheral termination or perhaps consumed throughout the entire extent of the axone. The active turnover of PNA in white matter has not been previously reported. Biochemical studies with S-35 methionine(15) have shown rapid incorporation into protein of spinal white matter in cats; in high resolution autoradiographs, this labeled protein was found chiefly in oligodendrocytes and in the glial septa enveloping myelin sheaths. Therefore, the interfascicular oligodendrocyte, as well as the nerve cell, appears to be a cellular site of active PNA and protein turnover. Since protein represents an important component of the myelin sheath(22), it is tempting to relate the nucleoprotein metabolism of interfascicular oligodendrocytes to myelin biosynthesis and to maintenance of the myelin sheaths in the mature animal. It is possible that neurological diseases characterized by demyelination reflect a disturbance in nucleoprotein metabolism of interfascicular oligodendrocytes.

Summary. 1. Adenine-8-C-14 and orotic-6-C-14 acid when injected intrathecally were rapidly incorporated into pentose nucleic acid of the central nervous system of mature cats. Once incorporated, these labeled precursors were retained for a relatively long time (51 days). 2. The pentose nucleic acid of spinal white matter and spinal nerve roots exhibited high specific activity, generally exceeding that of spinal gray matter and hindbrain. Spinal ganglia showed the lowest specific activity of pentose nucleic acid. 3. The deoxypentose nucleic acid showed insignificant activity in most experiments. When animals received repeated injections of labeled compounds and when they survived for longer periods of time (51 days), the deoxypentose nucleic acid fraction exhibited high activity; however, biochemical proof of incorporation is not yet available.

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α and β Lipoproteins and Serum Cholesterol Levels Following Administration of Unsaturated Fatty Acids. (23709)

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(Introduced by L. Freedman)
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Quantitative and qualitative alterations of ingested fat have been shown to regulate the composition of transported lipids in the human subject(1,2). Bronte-Stewart(3) postulated that atherosclerosis is possibly intensified by a dietary deficiency of essential fatty acids. The effect of lipotropic substances on dispersion of fat has been reported(3) and confirmed(4,5). The experimental data presented below indicate that serum alpha and beta lipoprotein and cholesterol levels are significantly modified by administration of essential fatty acids and lipotropic substances. The importance of these findings in the prevention of atherogenesis is discussed.

Material. Twenty-five patients, 16 males and 9 females, age 36 to 78, with clinical evidence of myocardial infarction, under our continuous observation for 4 to 5 years, were the subjects of this study. Controls were 20 subjects, ranging in age from 10 to 44 and one child of 3 (19 males and 2 females) with no demonstrable cardiovascular disease. The experimental as well as the control subjects were encouraged to remain on a normal diet with no restriction of fat, cholesterol-containing or any other food.

Methods. Determinations were done monthly on fasting serum of controls, experimental subjects and on pooled serum from healthy blood donors. Each blood sample was analyzed by a modified electrophoretic method for lipoprotein and protein fractions and by chemical analysis for cholesterol. The experimental subjects had been previously studied on an unrestricted diet while receiving a lipotropic mixture* and results reported(6). The same diet and lipotropic mixture were continued but to them was added 3 g of saf-

* Daily intake comprised 1 g of choline, 1 g of dl-methionine, 750 mg of inositol, 18 μg of vit. B_{12} and 750 mg of desiccated liver, supplied as Methischol Capsules, courtesy of U. S. Vitamin Corp., N. Y. City.