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Modification of Brain Deoxyribonucleic Acid Base Content with Maturation in Normal and Malnourished Rats

NATHAR W. PENN
New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, NY 10035, *U.S.A.*

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The mol percentage of 5-hydroxymethylcytosine is 2.2 times greater in the adult than in 2-day-old rat brain DNA. The concentration of 5-hydroxymethylcytosine falls in corresponding liver DNA preparations. This normal increase in brain 5-hydroxymethylcytosine is abolished in rats placed on an 8%-protein diet 5 days after birth.

The pyrimidine 5-hydroxymethylcytosine has been reported to characterize a central-nervous-system DNA species that is enzymically and chemically labile (Penn *et al.*, 1972). This DNA was implicated in higher functions of the central nervous system, presumably through modulation of synaptic activity by a fraction localized in the synaptosomal mitochondria (N. W. Penn, S. Schell, R. Suwalski & K. Bojanowski, unpublished work). In support of this interpretation, further study has demonstrated that the effects of barbiturate, alcohol and morphine are antagonized by DNA pyrimidines and their metabolically allied cofactors (Penn, 1974, 1975*a*,*b*). Time-intervals involved in reversal of drug action, barbiturate binding to DNA fractions and the specificity for DNA components as antagonists suggest a direct interaction between these drugs and a DNA receptor(s).

The acute actions of these drugs are mediated primarily or in part at the central-nervous-system synapse. It thus appeared that the DNA species characterized by 5-hydroxymethylcytosine, or the concentration of this base in DNA, might increase as the synaptic architecture of the brain was completed. We therefore investigated hydroxymethylcytosine concentrations in newborn and adult, normal and retarded rat brain DNA, by using liver DNA as a reference preparation.

Materials and Methods

The 8% (low-protein) diet for rats was purchased from ICN Life Sciences, Cleveland, OH, U.S.A. The original procedure for DNA isolation was used (Penn *et al.*, 1972) with the following modifications: the Sorvall model RC-2 centrifuge was used instead of the Spinco, but the same centrifugation schedule and *g* forces were maintained with the SS-34 rotor. Extractions of DNA were performed in polyethylene centrifuge tubes, with a radial clearance of 0.216mm between pestle and wall. The use of the uncapped semi-rigid centrifuge tubes used with the Sorvall rotor permits a more rapid processing of samples and slightly higher DNA yields. DNA bases, glutathione, iodoacetamide and lysozyme were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Sprague–Dawley adult rats of either sex, weighing 150–250g and accustomed to handling, were killed by guillotine. Brains were rapidly removed, weighed in tared beakers containing solution A (Penn *et al.*, 1972), and homogenized in solution A (Solution A/brain = 10:1, v/w). Brains from 2-day-old rats were similarly processed, combining tissue from two newborn animals for each sample. Livers from four 2-day-old animals, similarly prepared, were combined for each determination. To retard development, 5-day-old rats were placed on an 8%-protein diet, separated from the mother 2 weeks later and maintained on the diet until they were killed 2 months after birth. Animals from three litters were used.

The individual nucleotide bases were determined after formic acid hydrolysis. Appropriate portions were chromatographed in duplicate in propan-2-ol-/12M-HCl/water (170:41:39, by vol) (Wyatt, 1951) for precise evaluation of base ratios. Cytosine and 5-hydroxymethylcytosine were determined as total cytosines by this system, which does not resolve these pyrimidines. The ratio between the two components was determined by duplicate two-dimensional chromatographic runs in sodium acetate (0.1M, pH 3.5)/ethanol (1:4, v/v) (Loeb & Cohen, 1959) followed by butan-1-ol/0.1M-NH_3 (6:1, v/v) (Chargaff *et al.*, 1951). The bases were eluted with 0.1M-HCl and determined spectrophotometrically by the two-wavelength method (Bendich, 1957). The difference of 0.675 for 10μg of 5-hydroxymethylcytosine at 279.5 and 310nm was obtained from a reference spectrum (Venkstern & Baev, 1968). The amount of DNA was calculated on the basis of thymine, assumed to be 11% (w/w) of this nucleic acid in the rat (Zamenhof *et al.*, 1964).

Results and Discussion

The base content of newborn and adult brain and liver DNA species is given in Table 1. At death, animals on the 8%-protein diet averaged 71g in weight; age controls weighed an average of 164g. The variations in 5-hydroxymethylcytosine content between newborn and adult in these tissues are accompanied by corresponding opposite changes in cytosine content. These quantitative changes thus proceed within the restriction of the established ratios for the rat (Wyatt, 1951). No detectable RNA contamination is found in the samples, as indicated by these ratios, as well as by the absence of uracil from the chromatograms. In the DNA fractions isolated from brain, the content of 5-hydroxymethylcytosine rises sharply with maturation, whereas it falls in corresponding preparations from liver. Statistical analysis of the data by Student's t test for both tissues gives $P<0.01$ for the differences between newborn and adult values of 5-hydroxymethylcytosine. Constancy of total base ratios, but not of composition, is therefore a feature of brain DNA, and of corresponding DNA fractions from liver, during maturation. The increase in the percentage of 5-hydroxymethylcytosine in brain DNA and its decline in liver suggests that the DNA species characterized by this pyrimidine may bear a critical relation to the hyperplastic phase of development in the rat brain. The increase in cell number continues for 17 days *post partum* in this organ (Fish & Winick, 1969) but is substantially complete in the liver at birth.

The results shown in Table 2 indicate that 5-hydroxymethylcytosine metabolism in the brain is particularly sensitive to the developmental retardation induced by a protein-deficient diet. There is virtually no change in the concentration of this pyrimidine from the newborn value in the DNA of brain approx. 2 months after the animals have been placed on an 8%-protein diet. The nucleic acid containing 5-hydroxymethylcytosine may therefore be characterized descriptively as a 'developmental DNA'.

A number of reports have appeared which indicate that modifications in the structure of DNA may occur during the life cycle of the cell. In tissue culture, 5-methylcytosine is formed at DNA sites only after the synthesis of the macromolecule (Adams, 1974). In senescence, degradative changes such as the appearance of single-stranded regions in nuclear DNA of mouse tissues (Price *et al.*, 1971; Chetsanga *et al.*, 1975), increased damage to the nuclear DNA in the tissues of old rats (Samis *et al.*, 1966), lowered molecular weight of single-stranded DNA from rat liver nuclei (Massie *et al.*, 1972) or depletion of information from brain DNA (Johnson & Strehler, 1972) have been described. The present results indicate that other changes in DNA, whose significance remains to be assessed, may occur between these extremes in the life-span of the cell, both in brain and liver.

Analyses of purified tissue components may be misleading if the isolated preparations are not representative of the total material present. However, examination of the literature indicates the DNA content of the 48h-old rat brain to range from 1.6 to 1.75mg/g wet wt. (Mandel *et al.*, 1964; Fish & Winick, 1969). Our value of 1.62mg/g indicates that DNA isolation from newborn brain is virtually quantitative, and the 8.25% of 5-hydroxymethylcytosine in these samples therefore appears to be a valid estimate of its concentration in the bulk DNA. In the adult brain, DNA contents of 1.7–1.8mg/g have been reported (Fish & Winick, 1969; Chase *et al.*, 1969). This value is 0.7mg/g greater than the 1.1mg/g of adult DNA obtained in the present experiments. If, in the extreme case, it be assumed that the

Table 1. *Base composition of adult and newborn rat tissue DNA*

Formic acid hydrolysates were subjected to chromatography (in duplicate) as described in the text. Variation of individual values from means was ±4%. The numbers of samples are given in parentheses. Student's t test gives $P<0.01$ for the differences in 5-hydroxymethylcytosine content between newborn and adult rat DNA samples from liver and brain.

	Base composition (mol/100mol of DNA)			
	Brain		Liver	
	Newborn (6)	Adult (6)	Newborn (4)	Adult (5)
Adenine	28.6	28.9	29.0	28.3
Thymine	28.7	28.6	27.9	28.4
Guanine	21.4	21.6	21.8	21.8
Total cytosines	21.7	21.3	21.2	21.4
Cytosine	19.9	17.4	16.0	17.9
5-Hydroxymethylcytosine	1.79	3.94	5.2	3.5
5-Hydroxymethylcytosine (% of total cytosines)	8.25%	18.5%	24.5%	16.4%
DNA (mg/g wet wt.)	1.62	1.1	4.4	0.7

Table 2. *Effect of 8%-protein diet on brain DNA composition of 2-month-old rats*

The treatment of samples and the variation in the data were as described in Table 1. The numbers of animals are given in parentheses. Student's *t* test gives $P<0.01$ for the differences between 5-hydroxymethylcytosine content in animals on 8%-protein diet and in controls.

	Base composition (mol/100mol)		
		Normal diet	
	8%-protein diet (6)	Age controls (6)	Weight controls (6)
Adenine	28.9	28.9	28.1
Thymine	28.2	28.6	29.3
Guanine	21.6	21.6	21.2
Total cytosines	21.4	21.3	21.6
Cytosine	19.6	17.4	18.0
5-Hydroxymethylcytosine	1.84	3.94	3.61
5-Hydroxymethylcytosine (% of total cytosines)	8.64%	18.5%	16.7%

0.7mg of DNA/g lost in the isolation from adult brain contains no 5-hydroxymethylcytosine, then the actual percentage of 5-hydroxymethylcytosine would be $1.1/(1.1+0.7)\times 18.6\%$, or 11.3%. This value is greater by 37% than the 8.25% of 5-hydroxymethylcytosine in the newborn brain, and calculation shows this difference still yields a value of $P<0.01$. Should the 0.7mg that is not recovered contain some 5-hydroxymethylcytosine, then this 37% difference represents the minimal increase in the 5-hydroxymethylcytosine content of brain during maturation.

An analogous computation may be carried out for liver, but, since there is a low recovery of DNA from this tissue in the adult, no definite conclusion may be drawn. A value of 4.24mg of DNA/g has been reported (Euler & Hahn, 1948), but our method yields only 0.7mg/g. The true average percentage of 5-hydroxymethylcytosine in adult liver DNA would be determined by the content of the unrecovered fraction and may conceivably range from well below to above the newborn value. It can, however, be stated that there is a significant difference in 5-hydroxymethylcytosine content between the fractions isolated, and that these results do not suggest a parallelism in modification of DNA of brain and liver in the postnatal development of the rat. The results obtained from liver also show that this method for DNA isolation does not of itself lead to low values for 5-hydroxymethylcytosine in newborn tissue; $P<0.01$ is found for the higher content of 5-hydroxymethylcytosine in the liver DNA of the newborn compared with that of the adult. The lower 5-hydroxymethylcytosine content in newborn brain is thus not a procedural artifact.

The preparative method appears to be efficient in isolation of undegraded DNA in high yield from newborn animals. Extrapolation from published data (Campbell & Kosterlitz, 1950) gives 4.6mg of DNA/g of liver in the 2-day-old rat. Thus, in both newborn rat brain and liver, recovery approximates to previously reported maximal values. This may be related to the greater ease of homogenization of such tissues, or qualitative differences in the proteins associated with the DNA of very young animals (Kurtz & Sinex, 1967; Herrmann *et al.*, 1975).

A specific metabolic function cannot be ascribed to this DNA species at present. The requirement for DNA pyrimidines in countering the acute effects of hypnotic or sedative drugs and the absence of free 5-hydroxymethylcytosine from brain (N. W. Penn, unpublished observations) suggest that thymine, 5-hydroxymethylcytosine and deoxycytidine antagonize drug actions at a DNA site which modulates synaptic functions. This interpretation is supported by the specific displacement of barbiturate bound to DNA fractions by thymine and 5-hydroxymethylcytosine (Penn, 1975*a*) and the short time-intervals involved in antagonist action. Such a fraction of brain DNA would therefore belong to the category of DNA species characterized by rapid turnover (Iwamura, 1966; Gause *et al.*, 1973), is present in rat brain, and was reported to increase with maturation (Kimberlin *et al.*, 1974). This DNA species may be localized in part in the synaptosomal mitochondria, with a role in the maintenance of steady-state neuronal activity. The increased concentration of this DNA or its 5-hydroxymethylcytosine content during maturation of the central nervous system may be intimately related to the normal development of synaptic interconnexions and their behavioural correlates.

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