Deoxyribonucleic Acid Methylase of Mammalian Tissues*

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ABSTRACT: The existence of an enzyme, deoxyribonucleic acid methylase, which methylates deoxyribonucleic acid at the polymer level has been demonstrated in mammalian tissues.

The enzyme is located in the cell nuclei and during extraction it is obtained associated with the insoluble nuclear fraction. Cytosine is the only target base of the methylase. The enzyme is species specific and appears to have different levels of activity in different organs of the same animal. Some of the properties of the mammalian deoxyribonucleic acid methylase are described.

The methylated bases in DNA of microbial origin (6-methylaminopurine and 5-methylcytosine) have been shown to be acquired by the methylation of the preformed polymer by specific enzymes (Gold and Hurwitz, 1964; Fujimoto *et al.*, 1965). While it was reasonable to assume that 5-methylcytosine, whose distribution in DNA of mammalian sources is ubiquitous, is also acquired *via* the methylation of the polymer, attempts at the extraction of such an enzyme have proved fruitless. From a systematic search for such an enzyme the reason for its elusiveness became apparent. Unlike the DNA methylases from microbial sources which are readily solubilized by disruption of the cell, the DNA methylase of mammalian origin remains bound to an insoluble fraction of the nucleus. Moreover, enzyme activity can be demonstrated in the untreated pellet only within a very narrow range of protein concentration; slight excesses above the optimum concentrations were found to be highly inhibitory to the reaction.

It should be emphasized that in a search for these enzymes, DNA from heterologous sources must be used as the substrate. DNA from mammalian sources is fully methylated by its homologous enzymes *in vivo*, and therefore, *in vitro* interactions would yield but negligible incorporation. Such a heterologous interaction is adequate for the demonstration of the existence of the enzyme, but the number and distribution of methyl groups may not be related to *in vivo* homologous interactions.

Interaction of the DNA methylase from different organs of the same animal with the same recipient substrate revealed that the extent of methylation can vary extensively. Such variation in enzyme capacity implies a variation in the 5-methylcytosine content in DNAs of various tissues. A lack of homogeneity with respect to 5-methylcytosine content of DNA fractions from calf thymus has been previously noted from analytical data (Chargaff *et al.*, 1953).

Experimental Section

Isolation of DNA for Enzymatic Methylation. DNA from bacterial sources was isolated essentially by the method of Marmur (1961) with some minor modifications (Fujimoto *et al.*, 1965). DNA from rat liver and spleen was prepared by the method of Savitsky and Stand (1966). Salmon testes DNA and calf thymus DNA were purchased from Worthington Biochemical Corp., Freehold, N. J. The estimation of DNA was performed by the diphenylamine reaction (Burton, 1956).

Preparation of Nuclear Subfractions. Male and female rats of the Holtzman and Sherman strains weighing 90–120 g were used in most of the experiments. Where the DNA methylase activity was analyzed in a number of organs of the same animal, rats weighing 250–300 g were used. Regenerating liver was obtained from 100-g Sherman rats after partial hepatectomies (Higgins and Anderson, 1931). Two to three grams of the various organs were excised, carefully trimmed of fat and connective tissue, washed in ice-cold 0.25 M sucrose, and homogenized in four volumes of 0.32 M sucrose containing 3 mM $MgCl_2$ with a loose-fitting Ten Broeck homogenizer at 4°. Purified nuclei were then separated by the method of Pogo *et al.* (1966). In many of the experiments, the step which requires the homogenization of the nuclear pellet in 2.4 M sucrose was omitted, since it was found that it did not alter the outcome of the experiments. The purified nuclear pellet was then washed into a tight-fitting Ten Broeck homogenizer with 7 ml of a 0.02 M Tris–0.14 M NaCl–0.005 M $MgCl_2$ (pH 8.2) solution (Wang, 1963). The mixture was homogenized at high speed for 3 min at which time a milky foam appeared. The resultant suspension was centrifuged at 850*g* to eliminate unextractable nuclear material (I). The supernatant was then centrifuged at 2100*g* for 15 min at 4°. The floating lipid layer was

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pushed aside with a stirring rod; the supernatant was decanted and centrifuged at 105,000*g* for 60 min. The 2100*g* and 105,000*g* pellets were rehomogenized (1 ml/g of original organ) in 0.02 M Tris–0.005 M $MgCl_2$ (pH 8.2) buffer. The unextractable nuclear material (I) was again homogenized with 7 ml of 1 M NaCl–0.005 M $MgCl_2$–0.02 M Tris (pH 8.2) and was centrifuged at 2100*g* (II). The supernatant was centrifuged at 105,000*g* for 1 hr. The resultant pellet and the remaining unextractable nuclear material from the preceding 2100*g* centrifugation (II) were homogenized in 0.02 M Tris–0.005 M $MgCl_2$ (pH 8.2) buffer (1 ml/g of original organ).

For routine assays the nuclear homogenate obtained by extracting the nuclei with 0.14 M NaCl, 0.02 M Tris, and 0.005 M $MgCl_2$ was centrifuged directly at 2100*g*. A flow sheet in Figure 1 summarizes the fractionation method used for regular assays.

For the data presented in Table III the livers were homogenized with 0.25 M sucrose containing 2×10^{-4} M EDTA for the isolation of nuclei and the same sucrose–EDTA medium was also used for washing. The subsequent extraction of the enzyme from nuclei followed the procedure described earlier.

Preparation of Extranuclear Subcellular Fractions. Mitochondria, microsomes, ribosomes, and a soluble supernatant were prepared from rat liver after removal of the nuclear fraction essentially by the method of Schneider and Hogeboom (1950).

Assay of DNA Methylase Activity. Enzymatic activity was measured by determining the incorporation of [^{14}C]methyl groups into an acid-insoluble product which on hydrolysis yielded 5-methylcytosine. The incubation mixture consisted of 2 ml of 0.02 M Tris (pH 8.2), 0.02 ml (10 μg) of RNase, 0.25 ml of freshly prepared DNA substrate (0.5 mg), 0.1 ml of freshly prepared glutathione (10 μmoles), 0.025–0.4 ml of enzyme extract, and 0.02 ml (0.2 μc) of [methyl-^{14}C]*S*-adenosylmethionine, in a total volume of 3–4 ml. Blanks with either RNA or the enzyme extract omitted were used as controls. The incubations were carried out at 38° for 30 min. At this time another 0.01 ml (0.1 μc) of [methyl-^{14}C]*S*-adenosylmethionine was added and the incubations were continued for another 30 min. The tubes were then placed in an ice bath; DNA and enzyme extract were added to their respective assay blanks followed by the addition of 6 N HCl to all incubation mixtures to a final concentration of 0.2 N. After 30 min in the ice bath, the precipitate was centrifuged, 2 ml of 1.5 M hydroxylamine (pH 7.0) was added, and the mixture was incubated at 37° for 10 min. (The addition of this reagent lowers the blank values of incubations without enzyme, probably by the elimination of labeled SAM from the isolated DNA.) To the mixture was added 0.6 ml of 6 N HCl and 0.3 ml of 50% trichloroacetic acid with cooling for 15 min. The precipitate was then washed successively with 0.2 N HCl, 10% ethanol–0.2 N HCl, 50% ethanol–0.2 N HCl, 75% ethanol–0.2 N HCl, and alcohol–ether (3:1). The residual precipitate was washed twice with anhydrous ether, taken up in 0.2 N NH_4OH, and placed in planchets,

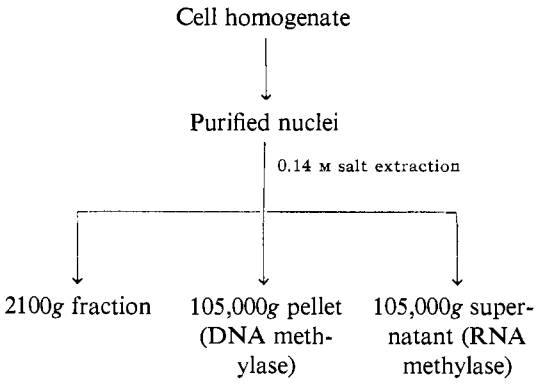

FIGURE 1: Subcellular localization of methylase activity in rat liver. The nuclei were extracted with 0.14 M NaCl–0.005 M $MgCl_2$–0.02 M Tris (pH 8.2). The 105,000*g* pellet contains the DNA methylase activity. RNA methylases are localized in the 105,000*g* supernatant which represents the bulk protein of the nucleus. RNA methylase was assayed by previously described methods.

and its radioactivity was determined in a Nuclear-Chicago low-background, end-window gas-flow counter (efficiency *ca.* 24%). For the data in Tables IV and V preliminary assays were performed to determine the saturation levels of enzyme extract, *i.e.*, further addition of extract produced no significant change in the total ^{14}C incorporated into DNA. The protein content of the enzyme extracts was measured by the method described by Lowry *et al.* (1951).

Pattern of Methylation. The washed precipitate obtained from the previous step was dried overnight *in vacuo* in a desiccator. (In some cases the incubation was terminated by the addition of an equal volume of phenol saturated with water and the mixture was shaken for 15 min and centrifuged. To the aqueous layer containing the DNA, trichloroacetic acid was added to give a final concentration of 5%. The precipitate obtained in this manner was then subjected to the washing procedures described earlier and desiccated.) The dry residue was then hydrolyzed in a sealed tube with 0.2 ml of 88% formic acid at 175° for 30 min in the presence of 0.5 μmole each of 6-methylaminopurine and 5-methylcytosine as carriers. The hydrolysates, after concentration to a small volume under a stream of nitrogen, were applied to Whatman No. 1 paper. The paper was then subjected to two-dimensional chromatography with butanol–0.2 N NH_4OH (6:1) in the first dimension for 18–24 hr followed by 2-propanol–HCl–H_2O (65:17.2:17.8) for 12 hr in the second dimension. The spots were located by ultraviolet light, cut into thin strips, and extracted with 2 ml of 0.05 N HCl at 37° for 18 hr. These solutions were transferred to stainless-steel planchets, dried, and counted. The 5-methylcytosine spot was at times subjected to further chromatography in butanol–acetic acid–H_2O (4:1:2), and its radioactivity was again determined.

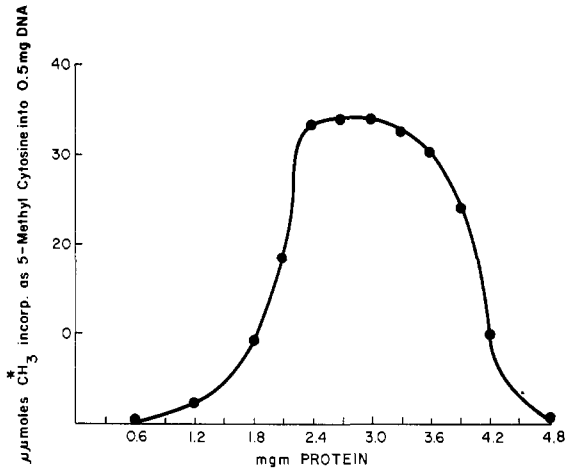

FIGURE 2: The activity of liver DNA methylase with increasing concentrations of protein.

Results and Discussion

In Table I the results of incubations of salmon testes DNA with extracts of various subcellular fractions of rat liver are presented. Only disrupted nuclei yielded extracts with unequivocal DNA methylase activity. The origin of the marginal activity observed with extracts of mitochondria is uncertain at present. These organelles may contain DNA methylases or the activity observed may have its origin in incomplete separation from nuclei. The resolution of this question awaits more exacting separations. The studies with the nuclear subfractions of rat liver as sources of DNA methylase are presented in Table II. Activity was found in the pellet obtained from centrifugation of 0.15 M NaCl extract, at 105,000*g*. This is the first example of an insoluble nucleic acid methylating enzyme. The soluble supernatant from this centrifugation contains some tRNA methylating capacity. However, the tRNA methylating activity of the nuclear fraction is only one-sixth to one-tenth of the total activity present in sucrose homogenates of whole liver (Srinivasan and Borek, 1964). Most of the tRNA methylating capacity is associated with the soluble supernatant obtained by centrifugation of the sucrose homogenate of the whole liver at 105,000*g*. Earlier investigations localized the tRNA methylases in the nucleoli of the germinating pea seedling (Birnstiel *et al.*, 1963) and Sirlin *et al.* (1963) also concluded from autoradiographic techniques that tRNA methylases are localized in the nucleolar part of the nuclei in salivary glands of *Smittia*. The difference in the behavior of the tRNA and DNA methylases may be due to the ease of solubilization of the former and its subsequent leakage from the nucleus during the sucrose homogenization of the whole liver. However, the exact localization of the tRNA methylases in the intact mammalian cell requires further study.

TABLE I: Subcellular Localization of DNA Methylase in Rat Liver.[a]

Subcellular fraction	CH_3 Incorp ($\mu\mu$moles) into DNA as 5-Methylcytosine
Sucrose homogenate (whole)	0
Nuclei	0
Nuclei (extract)	35
Mitochondria (extract)	7
Microsomes (extract)	0
Ribosomes (extract)	0
Soluble supernatant	0

[a] These data as well as those in Table II were obtained with the maximum activity of enzyme, where present.

TABLE II: Localization of DNA Methylase in the Nuclear Subfraction of Rat Liver Nuclei.

Nuclear Subfraction	CH_3 Incorp ($\mu\mu$moles) into DNA as 5-Methylcytosine
0.15 M NaCl Extract	
2100*g* pellet	2
105,000*g* pellet (membrane fraction)	35
105,000*g* supernatant (bulk protein)	0
1 M NaCl Extract	
105,000*g* pellet (DNA–histone)	0
105,000*g* supernatant	0
Unextracted Nuclei	
Residual chromatin and nucleoli	0

The activity of the mammalian DNA methylase with increasing concentration of protein is shown in Figure 2. At low protein concentration very little activity is seen. Between 1.2 and 2.4 mg the activity increases sharply and reaches a plateau which is maintained up to 3.7 mg of protein, and further addition of enzyme extract results in marked reduction of activity. This rapid decrease is probably due to the presence of some inhibitor. Falaschi and Kornberg (1965) have already shown that bacterial DNA methylase activity is easily inhibited by what appears to be a lipopolysaccharide. It is noteworthy that the reduction in methylase activity could be eliminated by the preparation of the original homogenate with 0.25 M sucrose containing 2×10^{-4} M EDTA. The DNA methylase activity of the fraction prepared by this modification is presented in Table III.

It should be emphasized that EDTA must be present

TABLE III: The Activity of DNA Methylase Extracted with Sucrose-EDTA.[a]

Amt of Enzyme Extract in mg of Protein in the Reaction Mixture	CH_3 Incorp ($\mu\mu$moles) into DNA as 5-Methylcytosine
0.4	12
0.8	30
1.6	64
2.4	66
3.2	60
4.0	58
4.8	58

[a] For details, see text.

during the extraction procedure for the suppression of the inhibitory effects in the preparation. If the reagent is added to reaction mixtures of enzyme preparations prepared without it the inhibitory effects of excess enzyme preparations are not diminished. This finding implies that EDTA may dissociate some inhibitory factors from the enzyme preparation.

The tRNA methylases of both bacterial and mammalian origin as well as the DNA methylases of bacterial origin were found to be species specific. It was therefore of interest to examine the possible species specificity of mammalian DNA methylase. In Table IV the interaction of various DNAs with DNA methylase from rat liver are presented. While heterologous methyl-

TABLE IV: Interaction of Heterologous DNAs with DNA Methylase from Rat Liver.[a]

Source of DNA	CH_3 Incorp ($\mu\mu$moles) as 5-Methylcytosine
Salmon testes	35
Micrococcus lysodeikticus	29
Shigella dysenteriae	18
Proteus vulgaris	0
Calf Thymus	12
Rat liver	0
Pseudomonas fluorescens	0
Rat spleen	0
Escherichia coli B	0
E. coli K_{12} W_6 (starved)	0
E. coli K_{12} W_6	0
Bacillus subtilis	24
Clostridium perfringens	0

[a] DNA (0.5 mg) and enzyme extract (0.2 ml) containing 2.4 mg of protein were used in all assays. 6-Methylcytosine was the only methylated base in the product.

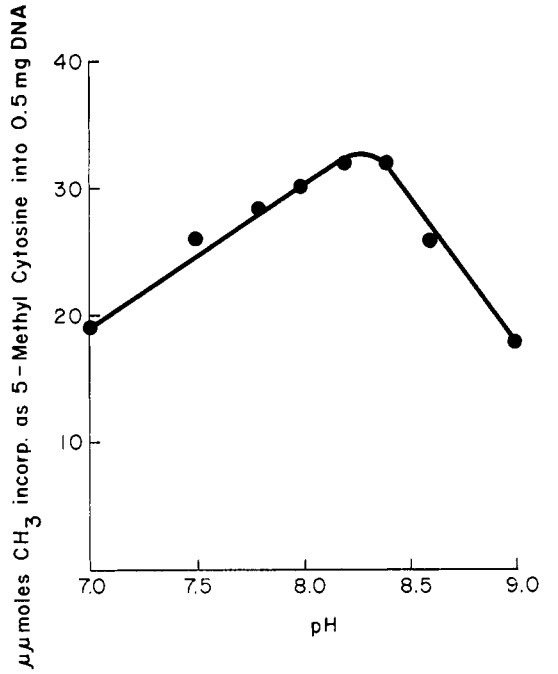

FIGURE 3: The pH optimum of DNA methylase.

ations are apparent, the DNA methylase from liver is more restricted than such enzymes of bacterial origin in its capacity to introduce supernumerary methyl groups into various DNAs. The homologous DNA was not methylated by the enzyme from the liver but DNA of calf thymus did serve as a recipient of methyl groups, albeit poorly.

In Table V the interaction of enzyme preparations from various organs with salmon testes DNA is presented. It should be emphasized that these reactions

TABLE V: Organ Distribution of DNA Methylase in the Rat.[a]

Source of Enzyme	CH_3 Incorp ($\mu\mu$moles) as 5-Methylcytosine into Salmon Testes DNA
Liver	35
Kidney	50
Brain	5
Spleen	6
Regenerating liver (24 hr)	32
Regenerating liver (72 hr)	32
Embryonic liver (18 days)	20
Embryonic liver (20 days)	20

[a] The fractionation procedure described in the text was used for each organ. The same insoluble nuclear fraction was used in each case at optimum activity.

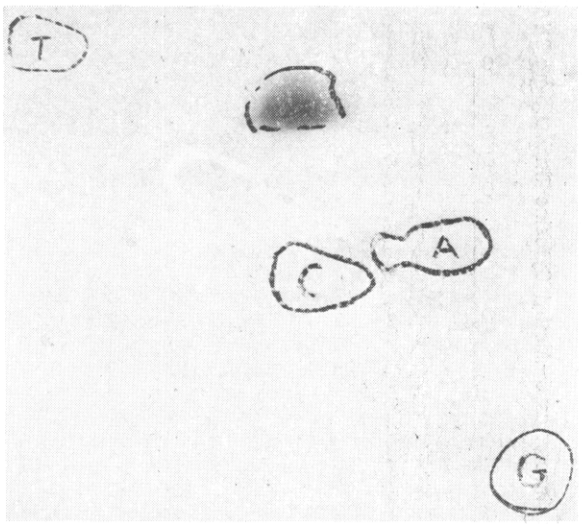

FIGURE 4: Radioautograph of hydrolysate of salmon testes DNA which had been methylated *in vitro* by mammalian DNA methylase. For method see text. For the method of hydrolysis and chromatography see Fujimoto *et al.* (1965). The areas of the major bases were transposed from the chromatography paper. The position of the radioactive area corresponds to that of an authentic sample of 5-methylcytosine.

TABLE VI: DNA Methylase in Various Organs of the Rat.[a]

Rat Organ(s)	Amt of Protein in Enzyme Extract (mg)	CH_3 Incorp ($\mu\mu$moles) as 5-Methylcytosine into DNA
Liver	1.8	30
Kidney	0.9	40
Liver and kidney	1.8 + 0.9	64
Liver	1.7	25
Spleen	1.4	3
Liver and spleen	1.7 + 1.4	28
Spleen	1.1	4
Kidney	1.1	33
Spleen and kidney	1.1 + 1.1	34

[a] Saturation levels of enzyme extract were not used in these experiments due to inhibition caused by increased amounts of protein. With the above protein concentrations approximately 60% of saturation activity level was obtained.

were carried out at saturation levels of enzymes. It is apparent that there are no differences between extracts from adult liver and regenerating liver. However, embryonic livers yielded extracts which are significantly lower in DNA methylating capacity. Extremely low levels of activity were observed in extracts of nuclei from brain and spleen. That the markedly lowered activity in extracts from the spleen is not due to the presence of some inhibitor was shown in experiments presented in Table VI. Admixture of extracts with high and low activities did not result in a reduction of methylating capacity.

The variations in enzyme capacity from different organs imply the possibility that the DNAs of those organs are not methylated to the same extent. Analysis of the 5-methylcytosine content of DNAs from these organs should prove to be of interest. Chargaff *et al.* (1953) have observed, in differential extracts of calf thymus DNA with salt, products with varying 5-methylcytosine content. Organ specificity of the enzymes which methylate tRNA has been demonstrated earlier by studies of the enzymes (Christman and Borek, 1967; Kay *et al.*, 1967) and by analysis of the methylated base contents of tRNAs (Bergquist and Matthews, 1962) from different organs.

The following properties of the DNA methylase of rat liver are worth recording. As can be seen from Figure 3 the pH optimum of the enzyme lies between 8.0 and 8.4 in Tris–HCl buffer. This is the preferred buffer for in phosphate buffer the activity was reduced by about 30%. Cytosine is the only target base for the enzyme (Figure 4). No metal requirement was observed nor was the activity inhibited by the presence of EDTA. Denaturation of DNA by heat and quick cooling eliminated the enzymatic activity completely as, of course, did DNase. When the heat-denatured DNA was annealed slowly, 80% of the activity was regained. The enzyme is quite labile. Storage at 4° for 1 day results in a decrease of 75% in activity. If frozen quickly and stored at −20°, the preparation retains 70% of its activity. However, on refreezing and thawing the activity falls to 15% of its original level.

The discovery of DNA methylase in a mammalian source opens the possibility of investigations of such enzymes under a variety of biological conditions. The results of such studies may provide an insight into the biological role of the methylation of DNA.

Added in Proof

A communication which appeared since the submission of this paper also locates the DNA methylase in an insoluble fraction of the mammalian nucleus (Burdon *et al.*, 1967).

References

Bergquist, P. L., and Matthews, R. E. F. (1962), *Biochem. J. 85*, 305.

Birnstiel, M. L., Fleissner, E., and Borek, E. (1963), *Science 142*, 1577.

Burdon, R. H., Martin, B. T., and Lal, B. M. (1967), *J. Mol. Biol. 28*, 357.

Burton, K. (1956), *Biochem. J. 62*, 315.

Chargaff, E., Crampton, C. F., and Lipschitz, R. (1953), *Nature 172*, 289.
Christman, J., and Borek, E. (1967), *Federation Proc. 26*, 867.
Falaschi, A., and Kornberg, A. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1713.
Fujimoto, D., Srinivasan, P. R., and Borek, E. (1965), *Biochemistry 4*, 2849.
Gold, M., and Hurwitz, J. (1964), *J. Biol. Chem. 239*, 3858.
Higgins, G. M., and Anderson, R. M. (1931), *Arch. Pathol. Lab. Med. 12*, 186.
Kay, A. M., Friedlander, B., and Leboy, P. S. (1967), *Abstr. 7th Intern. Congr. Biochem., Tokyo.*
Lowry, O. H., Rosebrough, N. Z., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marmur, J. (1961), *J. Mol. Biol. 3*, 209.
Pogo, A. O., Allfrey, V. G., and Mirsky, A. E. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 550.
Savitsky, J. P., and Stand, F. (1966), *Biochim. Biophys. Acta 114*, 419.
Schneider, W. C., and Hogeboom, G. H. (1950), *J. Biol. Chem. 183*, 123.
Sirlin, J. L., Jacob, J. and Tandler, C. J. (1963), *Biochem. J. 89*, 447.
Srinivasan, P. R., and Borek, E. (1964), *Federation Proc. 23*, 374.
Tsutsui, E., Srinivasan, P. R., and Borek, E. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1003.
Wang, T. (1963), *Biochim. Biophys. Acta 68*, 52.

Incorporation of Radioactivity from Labeled Serotonin and Tryptamine into Acid-Insoluble Material from Subcellular Fractions of Brain. I. The Nature of the Substrate*

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ABSTRACT: Incubation of ^{14}C-labeled 5-hydroxytryptamine (serotonin) or ^{14}C-labeled tryptamine with mitochondrial preparations from brain or liver leads to incorporation of radioactivity into acid-insoluble material obtained from the mixtures. This incorporation can be prevented by monoamine oxidase inhibitors. It is demonstrated by isolation, chemical synthesis, and nuclear magnetic resonance spectroscopy that the immediate substrates for the incorporation are indoleacetaldehydes. Incorporation is probably partly enzymatic and partly spontaneous. Enzymatic incorporation leads to material soluble in chloroform–methanol (2:1). This material was separated by Sephadex LH-20 filtration into five subfractions, and each subfraction was analyzed for a number of parameters. A similar incorporation occurs also *in vivo* after intraperitoneal administration of ^{14}C-labeled 5-hydroxytryptophan which is a precursor of serotonin.

During incubation of labeled 5-HT[1] with subcellular brain preparations radioactivity is incorporated into material insoluble in acid. This incorporation can be prevented by iproniazid and other MAO inhibitors (Alivisatos *et al.*, 1966b).

Evidence presented in this communication shows that the immediate precursor for the *in vitro* incorporation is 5-substituted indole-3-acetaldehyde. Current work on the isolation and identification of the product(s) of this reaction sequence(s) and evidence for its *in vivo* occurrence are also reported.

Materials and Methods

The majority of chemicals used in this study were commercial preparations (Alivisatos *et al.*, 1960, 1961). Indole-3-(ethylamine-2-^{14}C)bisuccinate (labeled tryptamine) was purchased from New England Nuclear Corp.

Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) was prepared from Tris according to Lewis (1966). It was recrystallized twice from water–ethanol; titrimetrically determined pK' was 6.5. Indole-3-acetaldehyde and indole-3-(acetaldehyde-2-^{14}C) were

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[1] Abbreviations used: DMSO-d_6, hexadeuteriodimethyl sulfoxide; TMS, tetramethylsilane; MAO, monoamine oxidase; 5-HT, 5-OH-tryptamine (serotonin); 5-HTP, 5-OH-tryptophan; NAD^+, oxidized nicotinamide–adenine dinucleotide; NADH, reduced NAD^+.