EFFECT OF MONOAMINE OXIDASE INHIBITORS ON THE LABELING OF SUBCELLULAR FRACTIONS OF BRAIN AND LIVER BY ^{14}C-SEROTONIN[1]

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We recently observed that under certain conditions, radioactivity from labeled serotonin becomes firmly associated with acid-insoluble material obtained from mitochondrial preparations. This association is prevented by MAO-inhibitors[3] (Alivisatos et al, 1966a). A preliminary report of our observations on the mechanism and the potential significance of such associations is presented here.

Methods: Nuclear, mitochondrial and microsomal fractions were obtained by homogenization of freshly excised and minced rat brain (2.5 g) in 0.25 M sucrose (25 ml) and subsequent differential centrifugation [20 minute periods at 700 x g (nuclei) and 12,000 x g (crude mitochondrial fraction containing nerve-endings; Whittaker, 1959); 60 minutes at 105,000 x g (microsomes)]. All fractions were washed twice by resuspension in 0.25 M sucrose and recentrifugation. The suspensions were adjusted to contain material corresponding to 5-10 mg of protein per ml. The latter was determined by the biuret method (Gornall et al, 1949) using bovine albumin as the standard. Subcellular fractions from liver were prepared in a similar manner. Incubations were as described in the legends of Figure 1 and Table I. At the end of the incubations, the mixtures were chilled, 50% trichloro-acetic acid was added dropwise with mixing to a final concentration of 8%. The precipitates were then washed several times by repeated solubilization in 0.1 N alkali and reprecipitation with trichloro-acetic acid (Liakopoulou and Alivisatos, 1965). The precipitate was then extracted 4 times with a mixture (Folch et al, 1957;

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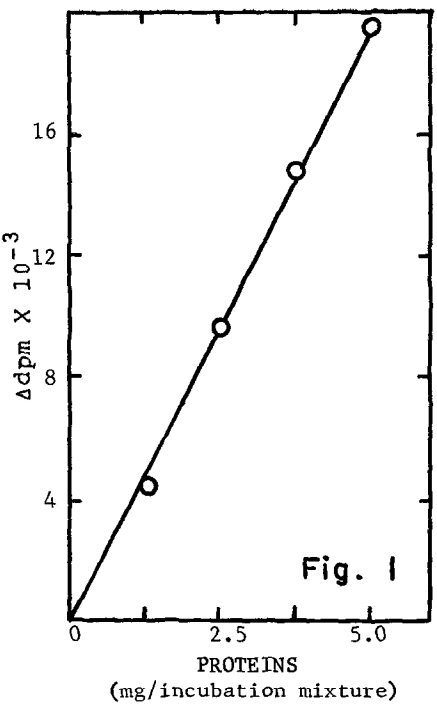
[3] Abbreviations: MAO monoamine oxidase; 5-HTP, 5-OH-tryptamine.

Robinson et al, 1965) composed of chloroform : methanol (2:1, v:v; 2 ml each time) and radioactivities were determined in the residue and in the combined organic solvent phase. Determinations in the scintillation counter were as described previously (Liakopoulou and Alivisatos, 1965). Disintegrations per minute (dpm) were computed from measurements of internal standards (^{14}C-toluene). Results were expressed as total radioactivities determined in the residue and in the organic solvent phase of each incubation-mixture minus the total radioactivities of the corresponding fractions of identically prepared zero-time controls (Δdpm). Controls were prepared ice-cold and were acidified immediately after addition of the radioactive serotonin. Acid-insoluble material obtained from such controls was invariably labeled. The extent of this "zero-time" labeling did not exceed 20% of the radioactivities measured in experimental samples after approximately 2 hours incubation at 37° (Table II).

Results: It was found that the mitochondrial fraction was most heavily labeled in comparison to the other fractions (Table I). Incorporation of radioactivity into the acid-insoluble material depended on the protein-content in the incubation-mixture (Fig. 1). A linear relationship also existed between the extent of labeling and the time of incubation within a 120 minute interval. Similarly, labeling increased in a linear fashion with increasing concentrations of the labeled precursor in the range between 0 and 10 mM. Approximately one-third of the total radioactivity in the acid-insoluble material was extractable by the chloroform-methanol mixture (Table I).

Labeled 5-HTP could be replaced by labeled tryptamine. However, when either 2(ring)-^{14}C-histamine or 3^{1}-^{14}C-5-OH-indolyl-3-acetic acid at 10 mM concentrations (0.2 μcurie, total) replaced labeled serotonin in the incubation-mixture, there was practically no incorporation of radioactivity in the acid-insoluble material. Nicotinamide-adenine-dinucleotide (10 mM) in the presence of 10 mM nicotinamide inhibited the incorporation by approx. 30%. Semicarbazide (5 mM) blocked completely the labeling of the acid insoluble material.

Iproniazid inhibited the incorporation of radioactivity into the acid-insoluble material obtained from mitochondria, but it had little effect in the nuclear and microsomal fractions (Table I). Other MAO inhibitors, including phenylisobutylhydrazine (0.03 mM), pheniprazine (0.03 mM), D-amphetamine (3 mM), p-trifluoromethylamphetamine (0.1 mM), p-S-methylamphetamine (0.1 mM), tranylcypromine (0.03 mM) and pargyline (0.03 mM) had similar effects. It was also demonstrated that during incubation serotonin is quantitatively converted to a product with different electrophoretic properties than those expected for either intact 5-OH-tryptamine or 5-OH-indolyl-3-acetic acid. Thus, at pH 8.0, 5-HTP migrates towards the negative elec-



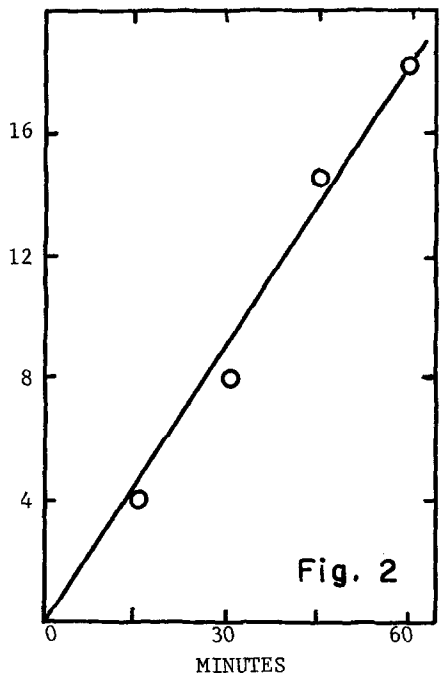


Fig. 1. Dependence on protein concentration of the incorporation of radioactivity from 3[1]-^{14}C-5-HTP into the acid-insoluble fraction from rat brain mitochondria. The reaction-mixtures (1 ml) contained 0.1 M Tris-HCl, pH 7.5; 60 mM KCl; 1 mM $MgCl_2$; 10 mM labeled 5-HTP (a total of 0.2 μcurie) and varying quantities of rat-brain mitochondria corresponding to the proteins indicated in the abscissa. Incubations for 2 hours at 37°. Termination of the incubation was as described in the text. Disintegrations per minute in the insoluble material were corrected by subtracting values obtained from identically prepared zero-time controls.

Fig. 2. Dependence on time of the incorporation of radioactivity from 3[1]-^{14}C-5-HTP into the acid-insoluble fraction from guinea pig liver mitochondria. Reaction mixtures as in Fig. 1, with 3 mg of protein per incubation mixture.

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trode (Samples Nos. 2, 3 and 4 in Table II), while 5-OH-indolyl-3-acetic acid moves in the opposite direction, covering approximately the same distance at any given time. The bulk of the radioactivity in the complete system (Table II, No. 1) remained at the origin. In this sample there was no detectable 5-HTP and only traces of 5-OH-indolyl-3-acetic acid.

Similar labeling of acid-insoluble material inhibitable by administration of MAO inhibitors was also observed *in vivo* (S.G.A. Alivisatos *et al*, 1966).

Discussion: The incorporation reported in this communication differs

TABLE I

Incorporation of Radioactivity from 3[1]-[14]C-5-Hydroxy-tryptamine into Acid-Insoluble Material Obtained from Subcellular Fractions of Rat Brain

Fraction	Radioactivity in Acid Insoluble Fraction (dpm)			
	Insoluble Material		Material Soluble in Organic Solvent	
	-	Iproniazid (1 mM)	-	Iproniazid (1 mM)
Nuclear (3.0)	4164	4614	1526	-653
Mitochondrial (5.2)	14432	4268	8351	-882
Microsomal (6.5)	4901	4784	6055	8490

Reaction mixtures identical to those in Figure 1. Values in parentheses indicate mg of protein per incubation mixture. When iproniazid (1 mM) was present, the mixtures were preincubated with the inhibitor for 10 minutes prior to the addition of serotonin. Termination of the incubation and determinations as described in the text. The dpm counted in the organic solvent-extractable material derived from the nuclear and mitochondrial fractions inhibited by iproniazid were less than the corresponding values of zero-time controls.

TABLE II

Electrophoretic Behaviour of Labeled Material Recovered from the Supernatant of Incubation-mixtures of [14]C-serotonin with Rat Brain Mitochondria

System	Maximum Electrophoretic Migration (cm)	Radioactivity in Acid Insoluble Material (dpm)
Complete	0	87,783
Complete, zero-time	11	2,711
Complete plus iproniazid (1 mM)	11	5,352
Complete, zero-time plus iproniazid (1 mM)	11	3,044

Reaction mixtures identical to those in Figure 1, with 6 mg of protein and 0.3 μcurie of total radioactivity per incubation mixture. At the end of the incubation period (zero to two hours) the samples were acidified with perchloric acid (5% final) and centrifuged. Excess perchloric acid was removed from the supernatants by neutralization with KOH in the cold and recentrifugation. Electrophoretic runs (2 hours, 18 volts/cm, 4°, TrisHCl at pH 8.0, Sp. resist. 200 ohms.) as described previously (Alivisatos et al, 1960). Migrations were measured from the middle (starting) line towards the negative electrode. The acid-insoluble material was prepared as described in the text, and extracted with organic solvent. Radioactivities correspond to the insoluble residue.

from previously studied associations of serotonin and other amines with either soluble proteins (Kerp and Kasemir, 1962) or blood platelets (Hughes and Brodie, 1959) or subcellular fractions from brain and other tissues (Robinson et al, 1965; Gillis and Giarman, 1964; Marchbanks et al, 1964). The prevalent mechanism of association reported in those investigations was that of cation-exchangers, involving intact 5-HTP or histamine as the cation. A method of choice for studying such exchanges has been equilibrium dialysis (Kerp and Kasemir, 1962; Marchbanks et al, 1964). In the present study labeled material was thoroughly washed from freely "exchangeable" label.

Our evidence indicates that the mechanism of this newly described association(s) most probably does not involve intact serotonin. Thus incorporation of radioactivity in acid-insoluble material of the various subcellular fractions parallels the known gross distribution of MAO (Zeller et al, 1955), i.e., it is most prominant in the crude mitochondrial preparations. Furthermore, a host of MAO inhibitors, including amines, hydrazines, etc., prevented, without exception, the incorporation at concentrations known to inhibit MAO-activity. Also, serotonin could not be replaced in this system by histamine which is a poor substrate for MAO (Weissbach et al, 1957; Blashko, 1963).

If MAO is involved in this process it is most probable that the reacting species is 5-hydroxy-indolyl-3-acetaldehyde. Additional support for this hypothesis is derived from the following observations: (a) the electrophoretic properties of the material found in the supernatant of our incubation mixtures are compatible with an aldehydic structure; (b) the weak inhibitory effect of NAD. The latter is required in the conversion of the aldehyde to its corresponding acid (Weissbach et al, 1957) and consequently reduces the concentration of the reactant. As mentioned before, 5-hydroxy-indolyl-3-acetic acid proved unsuitable as a labeling precursor in our studies. (c) The inhibitory action of semicarbazide in the labeling process may be attributed to an interaction with the aldehyde rather than with MAO. This view is based on the well-known insensitivity of MAO to semicarbazide (Zeller, 1963).

It is tempting to speculate that a probable mechanism of attachment involves condensation of the aldehyde with free amino groups of component(s) of the insoluble material leading to formation of Shiff bases:

In this equation the asterisk indicates the position of the ^{14}C-label and R stands for residues of presently unknown nature.

Whatever its mechanism, it is not known at present whether the incorporation *per se* is spontaneous or enzymatic. However, we do not believe that this type of association is involved in the storage and release of serotonin in tissues. From this standpoint and in view of the profuse occurence of this type of association *in vitro*, it is worthy to be taken into consideration by investigators concerned with the storage of intact biogenic amines. On the other hand, the *in vivo* occurence of such associations and their susceptibility to exogenous factors (drugs) (Alivisatos *et al*, 1966b) suggests that they may be involved in the mechanism of certain physiological actions of serotonin.

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