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

Spyridon G. A. Alivisatos† and Frieda Ungar

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^+.

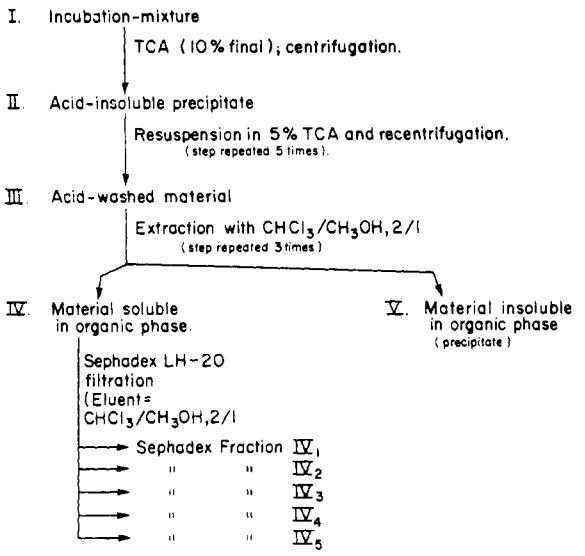

FIGURE 1: Fractionation scheme of incubation mixtures.

prepared from tryptamine and labeled tryptamine, respectively (Gray, 1959).

5-Hydroxyindole-3-(acetaldehyde-2-^{14}C) and indole-3-(acetaldehyde-2-^{14}C) were prepared enzymatically. Labeled serotonin (10 mM) or tryptamine (8 μc, total), 60 mM KCl, 0.1 M potassium phosphate (pH 7.5), 1 mM $MgCl_2$, and rat brain mitochondria (Alivisatos *et al.*, 1966b) corresponding to 94 mg of protein (Gornall *et al.*, 1949) were incubated in a 20-ml volume (75 min, 37°). The mixture was then chilled and centrifuged for 10 min at 25,000*g*. The supernatant was immersed in boiling water for 60 sec and recentrifuged as above. The clear, slightly yellow supernatant was kept frozen (solution A). The extent of conversion of indolamines to aldehydes was estimated from the distribution of the radioactivity on paper (Liakopoulou and Alivisatos, 1965) after electrophoresis at pH 8.0 (Alivisatos *et al.*, 1966b). It contained approximately equal amounts of intact indolamines and aldehydes. Only traces of acids could be detected. Virtual absence of acids was mostly due to absence of NAD^+ in the mitochondrial preparations derived from homogenates which possess high NADase activity (Mann and Quastel, 1941; Alivisatos and Dentsedt, 1951). The actual aldehyde content of various solutions was determined by the 2,4-dinitrophenylhydrazine method (Friedemann and Haugen, 1943), with synthetic indoleacetaldehyde freshly prepared from its bisulfite (Gray, 1959) as standard. Serotonin was determined according to Bogdanski *et al.* (1965). Preparative electrophoretic separation (Alivisatos and Woolley, 1956) of the indoleacetaldehydes from corresponding solutions A was in pyridine–acetate (pH 5.4), specific resistance 425 ohms at 4° (five paper strips, 5 v/cm, 16 hr). Under these conditions, intact indolamines and the corresponding acids move in opposite directions and escape at the end of the paper. Localization of the aldehyde was achieved by means of contact radioautography (Alivisatos *et al.*, 1963) and material in this zone was eluted with water.

Ascending paper chromatography in 1-butanol either saturated with ammonia (Gray, 1959) or with 3% formic (or acetic) acid(s) permits separation of the aldehyde from either the acid or the amine, respectively. The aldehyde moves in both instances with the front, while ionized molecules move much slower. Enzymatic preparations of indole-3-acetaldehyde showed traces of two impurities in the acid medium (R_F values 0.34 and 0.53), one of which was intact tryptamine. An unidentified impurity corresponding to 25% of the total was apparent in the medium saturated with ammonia (R_F 0.31).

Subcellular material prepared in 0.25 M sucrose from brain homogenates was either used directly or after preincubation with monoamine oxidase inhibitors. After incubation, the mixtures were acidified with trichloroacetic acid and the precipitates were treated as indicated in Figure 1. Fractions IV and V were most commonly used for radioactivity measurements in a scintillation spectrometer (Liakopoulou and Alivisatos, 1965). Disintegrations per minute were computed with the aid of internal standards (toluene-^{14}C) and corrected for identical zero-time controls.

Results

During incubation of labeled indolamines with subcellular preparations, radioactivity is incorporated in fractions IV and V (Table I). Most extensive incorporation occurs in the mitochondrial preparation. Residual incorporation observed in nuclei is probably due to contamination with nonnuclear material. No incorporation is observed in microsomal particles. Pargyline (*N*-benzyl-*N*-methyl-2-propynylamine hydrochloride, Table I), and other MAO inhibitors (Alivisatos *et al.*, 1966b), irrespective of structure, prevent incorporation. Histamine, which is a poor substrate for MAO, and indoleacetic acid gave negative results.

MAO is mainly localized in the mitochondria (Cotzias and Dole, 1951; Arioka and Tanimukai, 1957). The reactions catalyzed by this enzyme and by aldehyde dehydrogenase are as follows.

$$RCH_2NH_3^+ + H_2O + O_2 \xrightarrow{MAO} RCHO + NH_4^+ + H_2O_2 \quad (1)$$

$$RCHO + NAD^+ + OH^- \underset{\text{dehydrogenase}}{\overset{\text{aldehyde}}{\rightleftharpoons}} RCOO^- + NADH + H^+ \quad (2)$$

Our findings suggested that the probable precursor for this incorporation was the intermediate indole-3-acetaldehyde. This hypothesis was enhanced by the following observations. Crude solutions A or indoleacetaldehydes separated either chromatographically or electrophoretically could replace the indolamines in incubation mixtures. Pargyline could not prevent this

TABLE I: Incorporation of Radioactivity from Various ^{14}C-Labeled Compounds into Fractions IV and V Obtained from Subcellular Preparations of Rat Brain.[a]

Preparation[b]	Substrate	Pargyline (0.1 mM)	Radioactivity (Δdpm/mg of protein)		
			Fraction IV	Fraction V	Total
Nuclear (1.65)	Tryptamine	−	4,012	795	4,807
Nuclear (1.65)	Tryptamine	+	1,254	23	1,277
Mitochondrial (3.60)	Tryptamine	−	10,465	1,286	11,751
Mitochondrial (3.60)	Tryptamine	+	508	102	610
Microsomal (5.40)	Tryptamine	−	650	8	658
Microsomal (5.40)	Tryptamine	+	650	157	807
Mitochondrial (3.60)	5-Hydroxytryptamine	−	2,922	1,811	4,733
Mitochondrial (3.60)	5-Hydroxytryptamine	+	0	676	676
Mitochondrial (3.60)	Indoleacetic acid	−	0	22	22
Mitochondrial (3.60)	Histamine	−	0	340	340

[a] Mixtures contained subcellular fractions, 100 μmoles of Tris-HCl (pH 7.5), 50 μmoles of KCl, and 1 μmole of $MgCl_2$. Preincubation for 20 min at 37° in 0.5 ml in the presence or absence of 0.1 μmole of pargyline. After addition of 10 μmoles (0.2 μc) of either indole-3-(ethylamine-2-^{14}C) or 5-hydroxyindole-3-(ethylamine-2-^{14}C), or indole-3-(acetic acid-2-^{14}C) or imidazole-4(5)-ethylamine-2-^{14}C, incubation in 1 ml continued for 80 min. [b] Figures in parentheses indicate milligrams of protein per sample.

incorporation but it inhibited effectively the incorporation from indolamines used in tenfold concentrations (Table II). In these experiments in order to prevent utilization of intact indolamines present as contaminants, the mitochondrial preparations were routinely preincubated with a MAO inhibitor.

Tryptamine could also be replaced by chemically synthesized indole-3-(acetaldehyde-2-^{14}C) and the rate of incorporation of the latter was essentially identical with that of enzymatically prepared material (Figure 2). The identity of the two products was demonstrated as follows. Indole-3-(acetaldehyde-2-^{14}C) was obtained paper chromatographically from solution A prepared

TABLE II: Incorporation of Radioactivity from Electrophoretically Isolated 5-Hydroxyindole-3-(acetaldehyde-2-^{14}C) into Fractions IV and V Obtained from Crude Rat Brain Mitochondria.[a]

Substrate	Parglycin (0.2 mM)	Radioactivity (Δdpm/mg of protein)		
		Fraction IV	Fraction V	Total
Aldehyde	−	6508	2206	8714
Aldehyde	+	5942	2669	8611
Serotonin	−	7736	1801	9537
Serotonin	+	2692	236	2928

[a] Rat brain mitochondria (3.6 mg) and other additions, as in Figure 4, were preincubated for 20 min at 37°. Labeled substrate was then added and incubation at 37° was continued for 90 min. Aldehyde = 1 μmole (103,800 dpm) of material eluted from the origin (*i.e.*, middle part) of electropherograms; serotonin = 10 μmoles (222,000 dpm) of material eluted from the appropriate part of the same electropherograms.

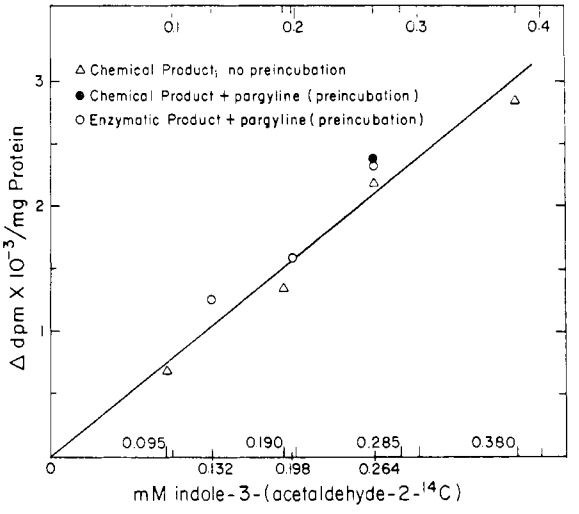

FIGURE 2: Incorporation of radioactivity into fraction IV from increasing concentrations of enzymatically (solution A) or chemically prepared indole-3-(acetaldehyde-2-^{14}C). When required, rat brain mitochondria (5 mg of protein) were preincubated with 0.48 μmole of pargyline and 100 μmoles of potassium phosphate (pH 7.5) in 0.4 ml for 30 min at 37°. Incubation in 1.2 ml for 60 min at 37°.

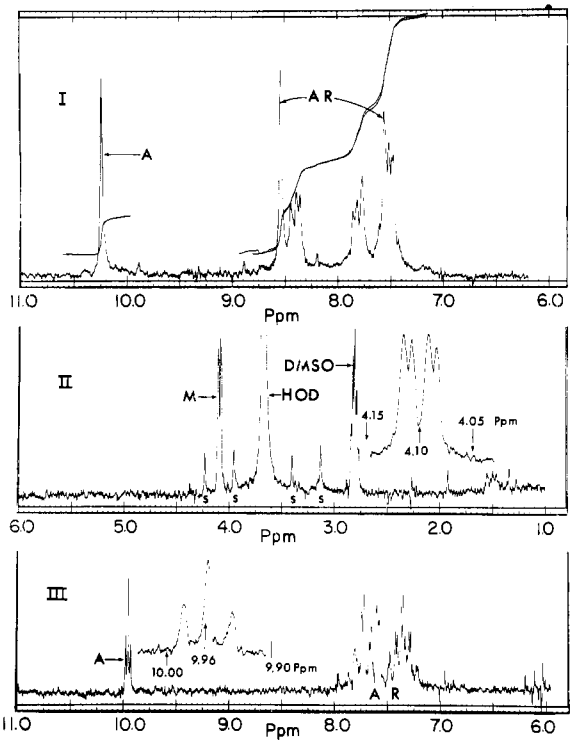

FIGURE 3: Nuclear magnetic resonance spectra of I, indole-3-carboxyaldehyde (~20 mg/0.4 ml); II and III, indole-3-acetaldehyde (~10 mg/0.4 ml). Both substances were dissolved in DMSO-d_6. Spectra recorded at 32° with a Varian HA-100 nuclear magnetic resonance spectrometer. The inserts in II (at 4.10 ppm) and in III (at 9.96 ppm) are tenfold expansions of the corresponding areas in the spectrum. Chemical shifts refer to TMS as an external reference. No corrections for bulk magnetic susceptibility, etc., were made. A = aldehydic proton; AR = protons in the indole ring system; M = methylene protons; and S = spinning side bands.

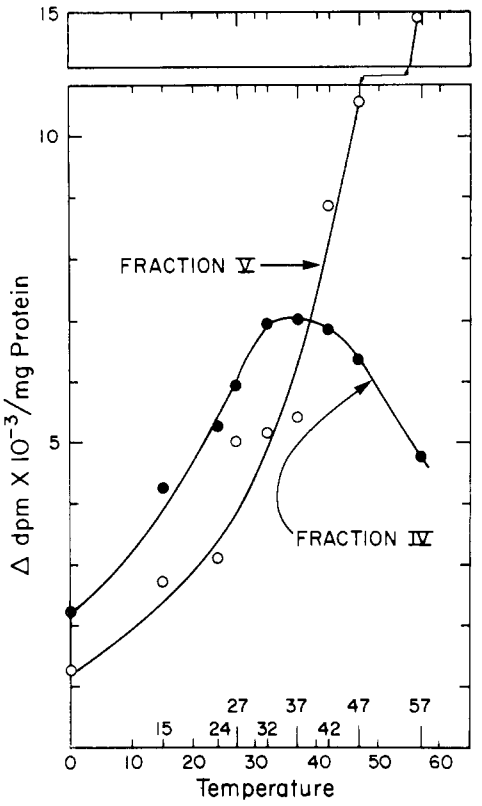

FIGURE 4: Temperature dependence of the incorporation of radioactivity from 5-hydroxyindole-3-(acetaldehyde-2-^{14}C) into fractions IV and V. Rat brain mitochondria (2 mg of protein), 15 μmoles of KCl, 0.5 μmole of $MgCl_2$, and 50 μmoles of potassium phosphate (pH 7.5) were preincubated with 0.2 μmole of pargyline in 0.25 ml at 37°. Incubations in 0.5 ml with solution A (~0.157 μmole of aldehyde-^{14}C, 8.88 × 10^4 dpm/μmole) at the indicated temperatures for 30 min.

from 30 μmoles of labeled tryptamine (150 μc). Material in a zone corresponding to R_F 0.8–1.0 was eluted with water. The eluate was freeze dried and extracted with peroxide-free dry ethyl ether containing 300 μmoles of synthetic indole-3-acetaldehyde prepared freshly from its bisulfite. After evaporation, the material was taken up in 10 ml of benzene, and 2 ml of saturated sodium bisulfite was added. After vigorous mixing the biphasic mixture was filtered. The filter paper was washed with ethanol and ether (Gray, 1959) and the bisulfite of the aldehyde was dissolved in water. The ratio of radioactivity to A_{280} of this solution (specific radioactivity) was measured, the volume was reduced, and nine volumes of ethanol were added. After 24 hr in the cold, the suspension was filtered. Material on the filter paper was dissolved in water, the specific radioactivity was measured, and recrystallization from 90% alcohol was repeated three more times. After the second recrystallization, the specific radioactivity of the solution remained constant (approximately 5000 dpm/μmole).

The nuclear magnetic resonance spectral characteristics of synthetic material are shown in Figure 3. Strip I, in the upper part of this figure, represents the spectrum of indole-3-carboxyaldehyde and it is given for comparison. The aldehydic proton of this compound gives a well-defined peak beyond 10 ppm downfield from external TMS. Strips II and III are parts of a single spectrum, *i.e.*, of indole-3-acetaldehyde. The signal due to the aldehydic proton (A) is also found at approximately 10 ppm downfield. However, it appears as a triplet due to coupling with the methylene protons (M) in the acetaldehyde side chain (J = 2.3 cycles/sec). The signal due to the latter protons (M) appears as a doublet at 4.10 ppm downfield. Coupling of the methylene protons (M) with the proton in the α position of the indole ring (J = 0.7 cycles/sec) results in further splitting of the peaks of the doublet created by coupling with the aldehydic proton. This is apparent in the tenfold expanded insert at approximately 4.10 ppm down-

TABLE III: Incorporation of Radioactivity from 5-Hydroxyindole-3-(acetaldehyde-2-^{14}C) into Fractions IV and V Obtained from Rat Brain Mitochondria and from Various Proteins.[a]

System	Radioactivity (Δdpm/mg of protein)	
	Fraction IV	Fraction V
A. Rat brain mitochondria	8,529	3,001
Versatol[b]	2,763	971
Bovine albumin[c]	3,567	97
Egg albumin[d]	3,786	1,063
α-Chymotrypsin[e]	1,808	1,287
B. Rat brain mitochondria	13,486	3,776
Boiled rat brain mitochondria[f]	5,091	5,901

[a] Mixtures contained various proteins or rat brain mitochondria corresponding to 2 and 1.3 mg per sample in expt A and B, respectively. Other conditions and preincubation as in Figure 4. After addition of labeled 5-OH-indole-3-acetaldehyde (0.15 μmole, 134,750 dpm) incubation in 0.5 ml at 37° was for 30 min. [b] A commercial mixture of human serum proteins (Warner-Chilcott Diagnostics). [c] Fraction V from bovine plasma (Armour Laboratories). [d] Fisher Scientific Co. [e] Armour Laboratories no. 148. [f] Immersed in boiling water for 60 sec.

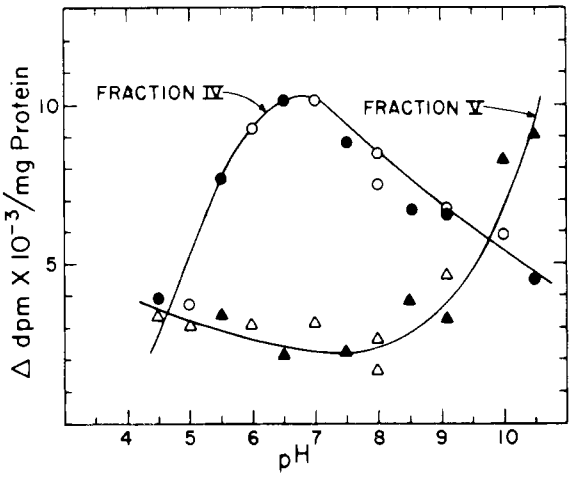

FIGURE 5: pH dependence of the incorporation of radioactivity from 5-OH-indole-(3-acetaldehyde-2-^{14}C) into fractions IV and V. Conditions as in Figure 4, with rat brain mitochondria equivalent to 1.85 mg of protein and the buffer was replaced by either 0.2 M sodium acetate (pH 4.5–5.5) or potassium phosphate (pH 6.5–7.5) or sodium carbonate (pH 8.5–10.5). Incubation at 37° for 30 min. Solid and open symbols correspond to two separate experiments.

field. The signal due to the α-proton is not specifically singled out in Figure 3, but it is located in the general area (7.20–7.85 ppm) of signals due to the protons of the indole ring system (AR) (Cohen *et al.*, 1960).

Once the nature of the substrates was established, we studied the mechanism of incorporation from the aldehydes. Dependence on time (0–120 min), the concentration of the aldehydes (0.1–1 mM), and the concentration of proteins (0.6–3 mg) were all shown to be linear for both fractions. In fraction V, however, the rate declines slightly at high concentrations of the aldehydes and in time-dependence studies. The dependence of incorporation on temperature is different in the two fractions (Figure 4). Incorporation in fraction IV passes clearly through a maximum in the vicinity of 37°. Incorporation in fraction V increases continuously with increasing temperature. A similar pattern is obtained in studies of pH-dependence curves (Figure 5). The curve for fraction IV shows a well-defined maximum in the vicinity of pH 7.0, while the curve for fraction V rises continuously with increasing pH above pH 8.0.

These observations suggest two different mechanisms of incorporation: one *enzymatic* for material soluble in the organic solvent and another *spontaneous* for insoluble material. This possibility is also supported by denaturation studies. Thus, incorporation into fraction IV decreased by 63% when boiled mitochondrial preparations were used while incorporation into fraction V increased by 35% (Table III). The latter would be expected from random unraveling and exposure of previously inaccessible groups in the molecule(s) of the receptor(s). Boiling, however, cannot eliminate completely incorporation in fraction IV. This is probably due to increased solubility in the organic phase (due to association of receptor molecules with indole residues) of some of the material that would otherwise be included in fraction V. The above argument is pertinent for incorporation occurring in the presence of diverse soluble proteins in the absence of rat brain mitochondria (Table III). It is possible that incorporation in such instances is only spontaneous.

Omission of Mg^{2+} or replacement of the latter by Ca^{2+} or the presence of 0.5 mM dipotassium ethylenediaminetetraacetate in incubation mixtures did not alter the pattern or degree of incorporation. The presence of *p*-mercuribenzoate (0.2 mM) had no effect.

Incorporation was inhibited by Tris (0.2 M, pH 7.5) in both fractions by about 60%, as compared to systems with 0.2 M potassium phosphate (pH 7.5). Bis-Tris (0.2 M, pH 6.5) was less inhibitory (30% as compared to systems with 0.2 M potassium phosphate, pH 6.5). The latter results favor a previously suggested hypothesis (Alivisatos *et al.*, 1966b) that incorporation may involve Schiff base formation by interaction of indolealdehydes with amino groups in the acceptor molecules.

TABLE IV: Analytical Data of Fractions IV_1–IV_5.[a]

Fraction	Radioactivity (dpm/ml)	μM[b]	A_{276} Obsd	A_{276} Expected from Radioactivity[c]	A_{276} Ratio[d]	A_{420}[e]	Esterified Phosphorus μM	Esterified Phosphorus P/R[f]	Dry Wt (mg/ml)	Proteins (biuret) (mg/ml)
IV_1	10,727	129	1.321	0.672	1.97	0.159	995	7.71	0.732	0.41
IV_2	5,683	68	0.817	0.355	2.28	0.100	1,140	16.76	0.895	0.21
IV_3	5,843	70	0.875	0.365	2.40	0.111	132	1.89	0.563	0.35
IV_4	3,130	38	0.500	0.198	2.61	0.075	240	6.47	0.593	0.40
IV_5	2,222	27	0.331	0.141	2.35	0.041	108	4.00	0.219	0.14

[a] These data correspond to the peak subfractions (5.0 ml) indicated by arrows in Figure 6. [b] Concentration calculated from the known specific radioactivity of the substrate (0.0375 c/mole). [c] Calculated under the assumption that the ϵ_{276} of 5-OH-tryptamine (approximately 5200) remained unchanged. [d] Observed per expected ultraviolet. [e] Indicates the intensity of the yellow-brownish color of individual fractions. [f] Micromoles of phosphorus per micromoles calculated from radioactivity as in *b*.

Work on the nature of the product(s) is presently limited to material soluble in chloroform–methanol (2:1) (Folch *et al.*, 1957). This material was filtered through Sephadex LH-20. The first subfraction (IV_1, Figure 6) is a mixture of entities with molecular weights above 4000 (upper limit of retention of LH-20). Subfractions IV_2–IV_5 are also mixtures but they probably correspond to entities of progressively smaller molecular weights. Material corresponding to each peak shows a constant ratio of A_{276} to radioactivity (Table IV). This ratio (~2), however, is higher than would be expected from radioactivity measurements and the known specific radioactivity of 5-hydroxyindole-3-acetaldehyde used during incubations. General characteristics of ultraviolet spectral patterns of individual subfractions (as compared to serotonin) are increased absorption at larger wavelengths, commensurate with the slightly dark coloration of these materials, and a shoulder at 305 mμ. All subfractions are biuret positive, but the presence of 5-hydroxyindole residues makes this test unreliable. Amino acid analyses of acid hydrolysates of the subfractions revealed the presence of 15 different amino acids in more or less similar proportions (Table V). Lysine residues, which could possibly qualify as potential acceptors, comprised 7–11% of the total. The hydrolysates also contained five unidentified peaks of which three (following valine, phenylalanine, and ammonia, respectively) appeared with much regularity in amino acid analyses. All fractions also contained esterified phosphate (Table IV).

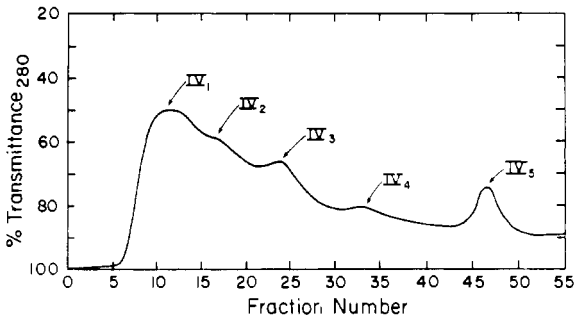

FIGURE 6: Elution pattern of fraction IV from a 160 × 2 cm Sephadex LH-20 column at 4–6°. $CHCl_3$–MeOH (2:1). Automatically registered elution curve through 0.3-cm light path. Rate of flow, 0.25 ml/min. Fraction volume was 28 ml between fractions 0 and 5 and 5 ml between fractions 5 and 55. Fraction IV was prepared from an incubation mixture with 5-OH-indole-3-(acetaldehyde-2-^{14}C).

A very important question is whether such reactions occur *in vivo* (S. G. A. Alivisatos, F. Ungar, S. S. Parmar, and P. K. Seth, in preparation). An experiment demonstrating the occurrence of incorporation in mouse brain after intraperitoneal injection[2] of DL-5-hydroxytryptophan-3^1-^{14}C and its prevention by pargyline pretreatment is presented in Table VI.

Discussion

The present studies are not related to the "binding" mechanism of intact 5-HT (Alivisatos *et al.*, 1966b; Marchbanks, 1966). However, the "medium affinity binding" of 5-HT reported by Marchbanks (1966) may be identical with the associations reported here.

[2] Only the L form of 5-HTP contributed directly in the *in vivo* formation of 5-HT (Freter *et al.*, 1957; however, see also Arendt *et al.*, 1966a,b). In general experimentation by intraperitoneal injection of 5-HTP is both costly and inefficient. In our more recent experiments labeled 5-HT of high specific radioactivity is injected endocranially–intraventricularly into mice. The reproducibility of such *in vivo* experiments approaches its *in vitro* counterparts (S. G. A. Alivisatos, F. Ungar, S. S. Parmar, and P. K. Seth, in preparation).

TABLE V: Amino Acid Composition of Acid Hydrolysates from Subfractions IV_1–IV_5.[a]

	Residue Per Cent[b]				
	IV_1	IV_2	IV_3	IV_4	IV_5
Aspartic acid	7.0	8.2	7.8	7.7	8.3
Threonine	6.5	6.5	6.8	6.9	6.7
Serine	10.6	9.0	11.6	12.0	11.2
Glutamic acid	5.4	8.2	7.1	6.6	6.9
Proline	3.8	3.8	3.7	3.8	2.9
Glycine	12.2	12.6	13.5	11.3	14.8
Alanine	7.0	5.0	7.9	8.0	7.1
Valine	4.4	4.6	5.2	4.7	4.8
Isoleucine	5.2	5.0	4.9	3.7	4.3
Leucine	10.3	9.0	10.5	9.9	9.5
Tyrosine	4.1	2.2	2.7	3.7	3.3
Phenylalanine	6.7	5.7	6.0	5.8	5.5
NH_3[c]	...	...	...	...	...
Lysine	7.0	11.3	5.6	8.8	7.6
Histidine	2.7	6.7	2.3	2.6	3.1
Arginine	7.1	5.0	4.9	4.7	4.1

[a] Each sample contained material from three consecutive tubes corresponding to a peak of the chromatogram in Figure 6. Samples were dried and hydrolyzed in sealed ampoules with 2 ml of 6 N HCl at 104°. Insoluble residue was discarded and amino acid composition was determined in a Technicon AutoAnalyzer. [b] Amino acid residues per cent were determined on the basis of total amino acids present in the sample. This total was on the average 0.333 μmole. [c] Ammonia peaks were always present but were not evaluated quantitatively.

In this respect, our findings should be considered in future studies of "intact" 5-HT binding. Depending on the methodology, it is conceivable that a smaller or larger part of reportedly "bound" 5-HT could be due to an incorporation similar to that encountered here.

Associations described in this paper also differ from previously described calcium ion dependent incorporations through transamidation of various amines, including histamine (Clarke *et al.*, 1959; Mycek and Waelsch, 1960) and histamine ribonucleoside (Alivisatos *et al.*, 1966c).

The incorporation reported in this study, on the other hand, shows marked similarities to phenomena observed by Pugh and Quastel (1937), Blaschko and Hellman (1953), and Nakai (1958) regarding an amine oxidase dependent "pigment formation." Nakai (1958) suggested that the low excretion of 5-hydroxyindolacetic acid following administration of 5-HT observed by Erspamer (1955) may be explained by *in vivo* pigment formation. Labeled material detected by radioautography after intraventricular injection of tritiated 5-HT in rat brain (Aghajanian *et al.*, 1966) is probably of the same nature (S. G. A. Alivisatos, F. Ungar, S. S. Parmar, and P. K. Seth, in preparation).

The present studies *prove* that indoleacetaldehydes are the immediate substrates for the incorporation observed in our laboratory. We have also shown that at least two different mechanisms exist, one of which is probably enzymatic and the other spontaneous. The nature of the products is not yet clear. These reactions may either involve incorporation into various acceptor molecules endowed with the proper residues (*e.g.*, free amino groups), or alternatively they may involve polymerization. In favor of incorporation are the excessive absorbance of partially purified material in relation to its radioactivity and the similarity of composition of peptide-bound amino acids present in all subfractions. In favor of a polymerization process is the similarity of spectral characteristics of subfractions with patently different molecular sizes. It is possible, however, that both processes occur simultaneously or that they are interdependent. Little is known regarding the composition of material insoluble in chloroform–methanol.

Of interest is the demonstration of the occurrence of such phenomena *in vivo*. The importance of this incorporation in modifying the properties of intracellular membranes (including their permeability to ions, etc.) cannot be assessed at present. However, it is tempting to speculate that results obtained in our laboratory regarding the inhibitory activity of indolamines in oxidative phosphorylation (Alivisatos *et al.*, 1964) may be directly related to such incorporations. Interesting from this viewpoint are observations[3] that preincubation of tyramine with submitochondrial preparations is necessary for maximum inhibition of the oxidation of succinate. This inhibition could be prevented by MAO inhibitors.

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TABLE VI: *In vivo* Incorporation of Radioactivity from 5-Hydroxytryptophan-3^{1}-^{14}C into Fractions IV and V Obtained from Mouse Brain and Liver Homogenates.

Organ	Pargyline (100 mg/kg)	Radioactivity[a] (dpm/g of wet tissue) Fraction IV	% Inhibn	Fraction V	% Inhibn
Brain	−	8,924	−	5,032	−
Brain	+	1,835	79.5	2,554	50.5
Liver	−	73,590	−	163,845	−
Liver	+	25,825	65.0	65,450	60.0

[a] Radioactivities are reported as dpm/g of liver or brain. They were calculated from the disintegrations per minute per milligram of protein in the homogenates. Young adult white mice were used. Pargyline was injected intraperitoneally to control animals 24 hr prior to the injection of labeled precursor (Everett *et al.*, 1963). DL-5-Hydroxytryptophan-3^{1}-^{14}C (Udenfriend *et al.*, 1957) (9 μmoles, 8406 μc) was injected intraperitoneally into each animal and identical injections were repeated twice at 1-hr intervals. All animals were sacrificed 2.5 hr after the last injection and the livers and brains were collected at 2–3° in 0.25 M sucrose containing 4 mM pargyline (prevention of postmortem incorporation). To aliquots of the homogenates 50% trichloroacetic acid was added to a final concentration of 10%. Fractions IV and V were obtained as described in Figure 1.

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