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EFFECT OF RESERPINE ON THE INCORPORATION *IN VIVO* OF RADIOACTIVITY FROM LABELED SEROTONIN AND OTHER 5-HYDROXY-INDOLE DERIVATIVES IN MOUSE BRAIN*

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Abstract—After endocranial-intraventricular administration of labeled serotonin or 5-hydroxy-indole-3-acetaldehyde into mice, radioactivity is incorporated into acid-insoluble material obtained from the brains of the animals.

Under proper experimental conditions pretreatment with pargyline diminishes the incorporation from serotonin, but incorporation from the aldehyde level remains unaffected.

Radioactivity due to acid-soluble metabolites obtained from the brain is higher after endocranial injection of serotonin into pargyline pretreated animals as compared to untreated controls. This effect is not observed after administration of the corresponding aldehyde.

Pretreatment with reserpine greatly increases the radioactivity due to soluble metabolites in the acid washings and of the incorporation both at the serotonin and the aldehyde levels (endocranial administration). This effect may be observed over a wide range of dosages. In reserpine-pretreated animals, increased radioactivity in the washings is also observed after endocranial injection of a variety of other labeled compounds, including tryptamine, DL-norepinephrine, dopamine, L-lysine and 5-hydroxy-indole-3-acetic acid. The possible mechanism of this effect is discussed.

PRETREATMENT with reserpine (i.p.) was recently shown to promote incorporation of radioactivity from exogenous labeled serotonin administered in the brains of mice.[1–3] Since *in vitro* incorporation, under our conditions, occurs almost exclusively after serotonin has been converted to its aldehyde derivative by monoamine oxidase,[4, 5] the effects of reserpine and MAOI§ on the incorporation after administration *in vivo*

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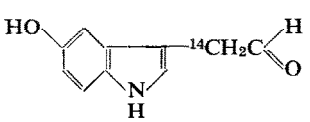
§ Abbreviations: MAO, monoamine oxidase; MAOI, monoamine oxidase inhibitors; 5HT, serotonin or 5-OH-indole-3-ethylamine; 5HIAAld, 5-OH-indole-3-acetaldehyde; 5HIAA, 5-OH-indole-3-acetic acid. Indication of position of isotopes in indole structures: (3[1-14C])-5HIAAld corresponds to:



of labeled 5-OH-indole-3-acetaldehyde (5HIAAld) and other substrates were also studied. These studies led to interesting conclusions with respect to the action of reserpine which are reported in this communication.

MATERIALS AND METHODS

Most of the chemicals (commercial) and methods used in the present work were described recently.[3, 5] Uniformly labeled ^{3}H-5HT (7100 c/mole) or (3^{1}-^{14}C)-5HT (56 c/mole) were purchased from Amerscham-Searle. (3^{1}-^{14}C)5HIAAld was prepared enzymatically from labeled serotonin.[6] (3^{1}-^{14}C)-5HIAA was prepared enzymatically.[7] This preparation was practically free of aldehyde (aliquots corresponding to 0·25 μmole of acid gave negative results with 2,4-dinitrophenylhydrazine[5]). Excess salts (phosphates) were removed at this stage by acidification of the watery solution to approximately pH 1·0 and re-uptake of 5HIAA in ether. The ether was removed by evaporation and the dry material was dissolved in H_2O and neutralized to pH 7·5. Alternatively, (3^{2}-^{14}C)-5HIAA (New England Nuclear Corp.) was used. Results obtained with either preparation were identical. Labeled precursors (i.e. indoleamines, indoleacetaldehydes, L-lysine, etc.) were given endocranially-intraventricularly to young adult male Swiss mice of approximately 30 g body weight.[3] The volume of injected material was always 0·02 ml and it contained 1·34 μmoles Tris HCl at pH 7·4. The animals were sacrificed at specified times after injection and the brains were excised, weighed and homogenized in 10% trichloroacetic acid (20 ml total volume) in a glass homogenizer equipped with a teflon-pestle. Precipitates obtained in this step were washed eight additional times with 10% trichloroacetic acid.[3] Radioactivities in the washed hyamine-solubilized precipitates and in the combined supernatants were determined in the scintillation counter as described previously.[3, 5] Counting efficiencies were determined with the aid of internal standards.

TABLE 1. EFFECT OF RESERPINE ON THE DISTRIBUTION OF RADIOACTIVITY IN MOUSE BRAIN AFTER ADMINISTRATION OF LABELED SEROTONIN*

Pretreatments	Dosage of 5HT (nmoles)	Radioactivity (dpm/g wet tissue in millions)	
		Acid soluble	Acid insoluble
1. —	5·0	2·50	0·06
2. Reserpine	5·0	3·50	0·08
3. —	10·0	3·82	0·11
4. Reserpine	10·0	6·17	0·14
5. —	25·0	6·25	0·21
6. Reserpine	25·0	13·74	0·33
7. —	50·0	22·76	0·70
8. Reserpine	50·0	29·90	1·12

* Mice were pretreated with reserpine (i.p., 5 mg/kg, 20 hr in advance). Uniformly labeled ^{3}H-serotonin (sp. radioactivity, 7·1 c/μmole) was injected in quantities shown in the table into the ventricular system, intracranially. Animals were sacrificed 30 min later. Processing of the brains was as described in the text. Each value represents analytical data obtained from pooled homogenates derived from three identically treated animals.

RESULTS

The dependence of radioactivity incorporation on the dosage of tritium-labeled 5HT is shown in Table 1. In such experiments the specific radioactivity and the volume of injected material remained constant. Incorporation increased with increasing dosage in the range from 5–250 nmoles (0·885–53·1 μg; Table 1 and Fig. 1).

As shown in Fig. 1, incorporation of radioactivity after intraventricular administration (250 nmoles) increases for the first 15–20 min after injection. A relatively flat

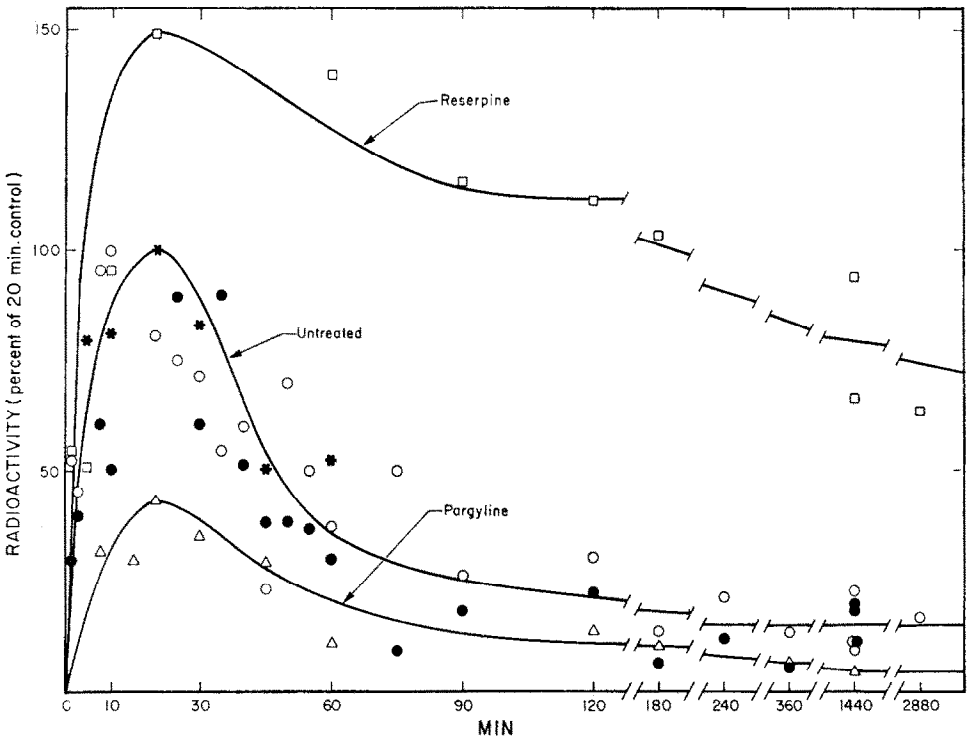


FIG. 1. Time-dependence of the incorporation *in vivo* of radioactivity into acid-insoluble material obtained from brain after endocranial-intraventricular injection of (3[1]-[14]C)5HT into mice. Mice were pretreated by intraperitoneal injection of either pargyline (100 mg/kg; 20 hr in advance) or reserpine (5 mg/kg; 20 hr in advance) and labeled serotonin [250 nmoles (0·50 μc)] was injected into the ventricles intracranially. Animals were sacrificed after this injection at times specified in the abscissa. Processing of the brains, etc. was as described in the text. Squares and triangles correspond to values obtained with reserpine or pargyline pretreated animals respectively. Light and dark circles and flower-like symbols correspond to values from three different experiments with untreated animals. Each symbol represents analytical data obtained from pooled homogenates derived from three animals. With materials of specific radioactivity 2 and in this range of dosages, maximum incorporation in untreated animals was of the order of 15,000 dpm per g brain.

maximum is eventually reached and, thereafter, incorporated material is gradually diminished. Under these conditions, however, residual radioactivity in the acid-insoluble precipitates from brain may be detected 4 or more days after injection. The general shape of this curve is not altered by pretreatment of the animals with either pargyline or reserpine. Nevertheless, at this dosage level incorporation is greatly diminished in animals pretreated with pargyline (Fig. 1).

Pretreatment with reserpine greatly enhances the incorporation *in vivo* and incorporated material remains at high levels long after the administration of labeled serotonin, practically for the duration of the manifestations of the reserpine syndrome (Fig. 1). Increased incorporation due to reserpine is observed over a very wide range of serotonin dosages (tested: 5 to 250 nmoles/animal with an average brain weight of 0·427 S.D. ± 0·039 g; see Table 1 and Fig. 1). This effect is also observed when the animals are kept in a constant temperature chamber so as to avoid body-temperature changes commonly observed in reserpinized small animals (Table 2). Furthermore,

TABLE 2. INCORPORATION *IN VIVO* OF RADIOACTIVITY IN MOUSE BRAIN AFTER ENDOCRANIAL-INTRAVENTRICULAR ADMINISTRATION OF ^{3}H-LABELED SEROTONIN*

No.	Pretreatment	Rectal temp. (C)	Radioactivity (dpm/g wet tissue in millions[9]) Acid soluble	Acid insoluble
1.	—	35–37·5†	12·12	0·15
2.	Reserpine (5 mg/kg)	25–27	17·05	0·21
3.	—	35–37·5†	9·71	0·28
4.	Reserpine (5 mg/kg)	35–37·5†	13·61	0·39
5.	Reserpine (10 mg/kg)	35–37·5†		0·44

* Mice were pretreated with reserpine (in doses indicated in the table, 20 hr earlier) and 10 nmoles (14·2 μc; 0·02 ml) labeled serotonin was injected intracranially. Processing and assays on pooled homogenates from brains of three identically treated animals were performed as indicated in Methods.

† Animals kept in an incubator maintained at 35° to obtain a rectal temperature of 35 to 37·5°.

the effect at constant temperatures is accentuated with increasing dosages of reserpine (Nos. 4 and 5, Table 2). However, the range of dosages of reserpine capable of producing such changes is rather narrow (2·5 to 12·5 mg/kg). These actions of reserpine are observed only as long as the cellular structure is preserved. Thus, if labeled serotonin is incubated *in vitro* with homogenates prepared from animals pretreated with reserpine (No. 2, Table 3), or with homogenates prepared from untreated animals but preincubated with relatively high concentrations of the drug (No. 3, Table 3), or finally with homogenates prepared from pretreated animals and preincubated with reserpine (No. 4, Table 3), there is no difference in the incorporation as compared to that of controls and in the absence of reserpine (No. 1, Table 3).

In all instances cited above increased incorporation due to pretreatment with reserpine was accomplished by increased amounts of soluble radioactive metabolites found in the brain 30 min after injection of the precursor (Tables 1 and 2). This was also true when labeled 5HIAAld was injected instead of serotonin (Nos. 5 and 6, Table 4). Thus, pretreatment with reserpine invariably results in increased radioactivity in the washings, irrespective of dosage (Table 1) or the nature of the labeled precursor (i.e. considering those compounds which were included in the present survey). For example, in addition to serotonin and 5HIAAld, similar elevations are

TABLE 3. INCORPORATION *IN VITRO* OF RADIOACTIVITY FROM LABELED (3[1]-^{14}C)-5-OH-TRYPTAMINE IN BRAIN HOMOGENATES IN THE PRESENCE OR ABSENCE OF RESERPINE*

Homogenate	Δdpm per mg of protein
1. From untreated animals	1563
2. From pretreated animals	1552
3. As in #1 with added reserpine†	1551
4. As in #2 with added reserpine†	1597

* Pretreatment with reserpine as in Fig. 1. Homogenates in 0·25 M sucrose were preincubated either in the absence (Nos. 1 and 2) or in the presence of reserpine (Nos. 3 and 4) for 30 min at 37° with 1 μmole $MgCl_2$ and 100 μmoles K-phosphate, pH 7·5. At the end of the preincubation (3[1]-^{14}C)-5-OH-tryptamine (10 μmoles, 0·1 μc) was added and incubation continued in a final volume of 1 ml, as above. After 2 hr, incubation was stopped by acidification with trichloroacetic acid and radioactivities in the acid-washed precipitates (Fraction III) were determined as described previously.[5] Values are differences of disintegrations per minute (dpm) of the experimentals and of zero-time controls. Such corrections amounted to less than 10 per cent of the total. Proteins were determined by the method of Lowry *et al.*[8]

† An aliquot (0·075 ml) of a 1:10 dilution in water of a commercial solution (Ciba Serpasil, 2·5 mg/ml) was added to the incubation mixture. Reserpine remaining in solution at the end of the incubation under these conditions corresponded to $1{\cdot}3 \times 10^{-8}$ moles/ml or approx. 43% of the original addition. This was determined in placebo experiments, i.e. in the absence of tissue homogenates. At the end of the incubation the placebos were filtered through SS 589 blue ribbon paper and reserpine in the filtrate was extracted in chloroform and determined spectrophotometrically (readings at 295 mμ; ϵ_{295} approx. 10,200).

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN MOUSE BRAIN AFTER ADMINISTRATION OF ^{14}C-LABELED 5-OH-INDOLE DERIVATIVES TO UNTREATED OR PRETREATED ANIMALS*

No.	Pretreatment	Injected derivatives	Radioactivity (dpm/g wet tissue in terms of 10^4)	
			Acid soluble	Acid insoluble
1.	—	5 HT	33·83	0·81
2.	Pargyline	5 HT	58·14	0·56
3.	Reserpine	5 HT	94·00	1·41
4.	—	5 HIAAld	27·90	4·43
5.	Pargyline	5 HIAAld	24·41	4·35
6.	Reserpine	5 HIAAld	62·84	7·69

* Mice were pretreated by intraperitoneal injection of either pargyline (100 mg/kg, given twice, 48 and 20 hr in advance) or reserpine (5 mg/kg, given 20 hr in advance). Either (3[1]-^{14}C)-5HT or (3[1]-^{14}C)-5HIAAld [25 nmoles of each (0·7 μc)] were injected intracranially. Animals were sacrificed 30 min later. Each value represents analytical data obtained from pooled homogenates derived from three identically treated animals.

TABLE 5. RADIOACTIVITY DUE TO ACID SOLUBLE METABOLITES PRESENT IN MOUSE BRAIN AFTER ADMINISTRATION OF LABELED COMPOUNDS*

No.	Pretreatment	Injected precursor (5 nmoles)	Radioactivity (dpm/g wet tissue in terms of 10^5) Acid soluble
1.	—	5HIAA†	0·04
2.	Reserpine	5HIAA	0·06
3.	—	Tryptamine‡	2·52
4.	Reserpine	Tryptamine	4·82
5.	—	DL-Norepinephrine§	16·07
6.	Reserpine	DL-Norepinephrine	22·49
7.	—	Dopamine‖	5·88
8.	Reserpine	Dopamine	10·17
9.	—	Lysine¶	10·78
10.	Reserpine	Lysine	15·75

* Experimental set-up was as in Table 1.
† 5HIAA (3^{2}-^{14}C) (New England Nuclear Corp.) sp. radioact., 1·88.
‡ Tryptamine-^{3}H-HCl (uniformly labeled) (Amersham/Searle) sp. radioact., 247.
§ DL-Norepinephrine-7-^{3}H acetate (New England Nuclear Corp.) sp. radioact., 500.
‖ Dopamine-^{3}H-HBr (New England Nuclear Corp.) sp. radioact., 500.
¶ L-Lysine HCl ^{14}C-uniformly labeled (Schwarz Bioresearch, Inc.) sp. radioact., 247.

TABLE 6. ELECTROPHORETIC SEPARATION OF SOLUBLE LABELED METABOLITES OF (3^{1}-^{14}C)-5-OH-TRYPTAMINE PRESENT IN MOUSE BRAIN HOMOGENATES*

Pretreatment	Applied radioactivity (cpm)	Total (cpm on paper)	Recovered radioactivities: Distribution (per cent) Serotonin	Neutral metabolites	5-OH-indole-3-acetic acid
1. Untreated	4821	3319(±68)	16·4(±0·91)	11·1(±0·66)	72·5(±0·189)
2. Pargyline	5122	4012	70·1	4·9	25·0
3. Reserpine	7659	5305	22·6	13·8	63·5

* Pretreatment of the animals and other experimental conditions as in Table 1, except that the labeled serotonin had a specific radioactivity of 11·4 c/mole. Excised brains were homogenized in 10 ml of 10% perchloric acid and centrifuged. Excess perchloric acid was removed from these mixtures by neutralization with KOH at 1° and volumes were reduced by lyophilization. Aliquots (0·03 ml) applied on the paper corresponded to radioactivities shown in the table (counting efficiencies were 60 per cent). Electrophoretic runs (225 min, 18 volts per cm, 4°, Na-acetate at pH 5 1, specific resist., 200 ohms at 4°) and detection of spots by radioautography were as described previously.[9] After film exposure, the appropriate areas of the paper were cut into 1·6 × 1·6 cm squares, set in vials containing scintillation fluid and counted as described previously.[10] Results are expressed in terms of percentages of actual counts per minute (cpm) corresponding to the area of each spot in relation to the total counts corresponding to all spots (detectable after 10 days' exposure in radioautography). This total recovered radioactivity (100 per cent) is given in the appropriate column in terms of actual cpm (approx. counting efficiencies were 50 per cent). Numbers in parentheses indicate standard deviations calculated from three different electrophoretic runs, in which aliquots (4821 cpm) from the homogenates of the untreated animal were included. They serve to demonstrate the reproducibility of the electrophoretic separations and of the recoveries under these conditions. The positions of serotonin and of 5-OH-indole-3-acetic acid were indicated by inclusion of authentic controls in each run.

observed with 5HIAA and a variety of other compounds, including tryptamine, DL-norepinephrine, dopamine and L-lysine (Table 5). It should be noted that, in contrast to the effect of pretreatment with pargyline, pretreatment with reserpine increases the radioactivity in the washings without affecting the normal distribution of soluble radioactive metabolites (Table 6).

Incorporation in untreated animals is, as expected,[3] much higher from the aldehyde as compared to the incorporation from serotonin (Table 4). In this range of dosages, pargyline diminishes the incorporation from serotonin (see also Fig. 1), while radioactivity in the washings is nearly doubled (Nos. 1 and 2, Table 4) and the composition of the pool of soluble radioactive metabolites is drastically changed (Table 6). Pretreatment with pargyline is ineffective, in the aspects mentioned above, if the aldehyde (instead of serotonin) is used as the precursor.

DISCUSSION

The mechanism of binding of biogenic amines or their derivatives is not clear. Probably, several types of binding exist, fulfilling different purposes. Thus, the work of Marchbanks points towards binding sites with vastly different affinities for intact serotonin.[11] Even at the same affinity range, different sensitivities of binding towards various neurotropic agents (i.e. reserpine or *d*-LSD) suggest different modes of attachment.[12] Similarly, recent work of DeRobertis *et al.*[13] points towards differences of attachment of *d*-tubocurarine and implicates the involvement of special proteolipids at the nerve endings.

Ideally, it should be possible to isolate such combinations in forms amenable to chemical characterization. In our past attempts[4, 5] we described methodology for the isolation of firmly bound material derived from biogenic amines incubated with tissue homogenates. This combination *in vitro* could be largely prevented by monoamine oxidase inhibitors.[4] Considering that isolation procedures may alter the chemical nature of a given combination, this incorporation[5] may be related to the *medium affinity* binding reported by Marchbanks.[11] The latter is also inhibited by MAOI.[11] These studies have further shown that under our conditions the immediate precursors for this enzymatic incorporation *in vitro* are *aldehydes* derived from the corresponding amines through the action of MAO present in the incubation mixtures.[5] Similar findings and conclusions were reported by Keglević *et al.*[14]

Other types of covalent bonding are also possible *in vitro*. They probably occur by spontaneous interaction of the intact amines with constituents of partially purified proteolipid preparations.[15] Most probably, in studies *in vitro*, it is necessary to suppress (or eliminate) enzymes involved in the incorporation from the aldehyde level in order to study the (spontaneous) incorporation of intact amines.

In recent studies *in vivo*[3] we applied the methodology used in our work *in vitro*[5] for the isolation of incorporated material. Incorporation could be *partly* prevented by pretreatment of the animals with MAOI. This (MAOI-sensitive) part of the incorporation *in vivo* (exogenous serotonin dosage-range 25 nmoles and above was attributed to the aldehyde.[3] Evidence reported here confirms this conclusion: Incorporation was much higher in experiments in which the aldehyde rather than the intact amine was given to animals and pretreatment with MAOI failed to inhibit incorporation from the aldehyde.

In the present study our primary interest concerns the mechanism of stimulation of the incorporation *in vivo* by reserpine.[3] Reserpine is completely ineffective in our

systems *in vitro*. However, pretreatment of the animals with this drug unfailingly increases the incorporation *in vivo* from exogenously administered labeled serotonin. This effect is observed over an exceptionally wide range of dosages, including quantities of the order of those normally encountered in the brain.[16] Such dosages are within the lower range of dosages currently used by other investigators.[17]

Increased incorporation in reserpine-pretreated animals is always accompanied by a considerable increase of the radioactivity due to soluble metabolites remaining in the brain at the time of sacrifice (usually, in our experiments, 30 min after injection of the labeled precursor). Furthermore, when serotonin is used as the precursor, although the overall radioactivity is elevated, there is no appreciable change of its distribution among the various soluble metabolites, as compared to the distribution in untreated animals (Table 6). It should be noted that the effects of reserpine with respect to incorporation and the overall radioactivity remaining in the washings are similar if labeled 5HIAA1d is given instead of serotonin. Considering that incorporation *in vivo* increases with increasing dosage (Table 1), it may be concluded that the effect of reserpine on the incorporation is secondary, its primary action being accumulation of metabolites in the soluble fraction.* This hypothesis was tested by using 5HIAA and a variety of labeled compounds, other than serotonin or its aldehyde. In all cases, radioactivity due to soluble metabolites in the brain 30 min after injection was significantly elevated in reserpine-pretreated animals as compared to untreated animals. Such results point towards a *general retardation* of the removal of small metabolites from the brain of mice after i.p. reserpine-pretreatment. This effect of reserpine is reminiscent of a similar retardation of the transfer of 5HIAA from the brain to plasma observed after pretreatment of the animals with probenecid.[19] The mechanism of this slow down of the removal of soluble metabolites from the brain by reserpine is presently unknown. It is, however, well-known that despite its antihypertensive properties, reserpine is devoid of any actions on cerebral blood flow or the metabolic rate in the brain of humans.[20] Drop of the body temperature observed in small animals pretreated with reserpine is an important contributing factor involved in many effects *in vivo* of reserpine.[21] In the present study it was shown that although retardation of the removal of soluble metabolites from the brain is accentuated in animals kept at room temperature, this effect is also observed in animals in which temperature is maintained at 37°. Differences in the experimental setup prevent direct comparison of our results with those of Lajtha and Toth[21] regarding the effects *in vitro* (slices) and *in vivo* of reserpine in the uptake of L-lysine. However, in both cases altered permeabilities at the cellular level must be responsible for the drug action.

Retardation of the removal of soluble radioactivity from the brain observed after administration of labeled serotonin to pargyline pretreated animals deserves a brief comment. This effect is definitely due to the MAOI activity of the drug: the composition of the radioactive pool is drastically changed with highly increased intact serotonin as its main constituent, while incorporation is diminished. In addition, this effect of pargyline is absent when precursors are used which are not substrates of monoamine oxidase, i.e. 5HIAA1d.

It should be noted that the overall clearance of serotonin from the body after *intraperitoneal* injection is not much changed by pretreatment of the animals with MAOI.[22]

* Interference with storage[18] also contributes in increased incorporation from the aldehyde level, especially when very small dosages of serotonin are used.

Since, in comparison, tryptamine clearance is very much retarded under these circumstances, it was concluded that glucuronide formation is the main alternative pathway facilitating clearance of serotonin.[22] In the present studies serotonin was given endocranially and electrophoretic patterns of radioactive metabolites present in the washings indicate that under our conditions glucuronide formation (in the brain) is insignificant. Evidently, increased radioactivity in the washings is due to a *reverse barrier* opposing the movement of intact serotonin from the brain to the systemic circulation.

The significance of 5HIAAld and its possible binding are not yet understood. Considering the localization of serotonin in the brain[23] and mounting evidence for its involvement in the "wet neurophysiology" of sleep,[24] it is tempting to speculate[25] that the aldehyde and its incorporation may be implicated in this complicated process. The observation by Jouvet *et al.*[26] that paradoxical sleep is eliminated for a long period by pretreatment with monoamine oxidase inhibitors further supports this view. It was actually suggested[27] that an unknown deaminated metabolite of serotonin may be responsible for the triggering of paradoxical sleep. Furthermore, reserpine pretreatment elicits a prolonged ponto-geniculo-occipital (PGO) activity in normal cats.[28] Increased incorporation from the aldehyde level and a slow down of the removal of incorporated material in reserpinized animals (Fig. 1) may indicate a cause–effect relationship of the incorporation to the PGO elevated activity. The latter is totally suppressed by MAOI.[29]

It was recently shown that in chicks 24–48 hr old (i.e. lacking effective blood–brain barrier) indole acetaldehydes induce sleep.[30,31] A study of the topographical localization of this type of incorporation in the brain and its intracellular distribution after administration of labeled precursors to experimental animals will undoubtedly help in the correlation of functional and biochemical aspects of this problem.

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