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DRUG-INDUCED ALTERATIONS IN THE SUB-CELLULAR DISTRIBUTION OF 5-HYDROXYTRYPTAMINE IN RAT'S BRAIN*†

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Abstract—High-speed centrifugation of preparations of brain (rat) has permitted the separation of two forms of 5-hydroxytryptamine (5-HT; serotonin), "bound" and "free", and the influence of drugs on these forms has been studied. Normally, about 30 per cent of the total endogenous 5-HT is found in the free form. Some drugs known to depress or tranquilize (such as reserpine, chlorpromazine, phenobarbital), irrespective of their effect on total cerebral 5-HT, produce significant increases in the proportion of free 5-HT. The hallucinogenic agent, LSD-25, and the anti-depressant drugs imipramine and β-phenyl*iso*propylhydrazine lead to an elevation in total 5-HT, which may be accounted for by greater increases in bound than free 5-HT. The anti-depressant drug, iproniazid, produces a rise in both forms of the amine with no significant change in distribution. Among the anti-depressants, only iproniazid completely prevents the release of granule-bound 5-HT which ordinarily is induced by reserpine. This action is not entirely related to inhibition of monoamine oxidase or to the high levels of 5-HT produced by iproniazid.

THE association of the bulk (70 per cent) of endogenous 5-hydroxytryptamine (5-HT; serotonin) with the particulate fraction of homogenates of the rat brain has been reported previously by Giarman and Schanberg.[1] A similar pattern of intracellular distribution of 5-HT has been reported by Baker[2] in duodenal mucosa of the rat, and by Walaszek and Abood[3] in the brain of the rat.

Studies in this and other laboratories of drug-induced alterations in the total level of 5-HT in whole brain or in specific regions of the brain have not demonstrated a trend consistent with simultaneous drug-induced behavioral changes. The purpose of this investigation was to determine whether a relationship exists between drug-induced alterations in the subcellular distribution of 5-HT and behavioral changes. This report indicates that there is a predictable differentiation of effect produced by neuropharmacologic agents which generally depress the central nervous system (CNS) and those which might be classed as anti-depressants, euphoriants, and psychotomimetic substances.

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MATERIALS AND METHODS

These studies were done on freshly excised brains (without olfactory lobes and pituitaries) of adult male rats, sacrificed by decapitation. All operations prior to centrifugation were carried out in a refrigerated room at 4 °C. Homogenates (1:10) of brain were prepared in a glass homogenizer with an aqueous medium containing sucrose (0·25 M), edathamil (0·002 M) and *p*-tolyl ether of choline (an inhibitor of monoamine oxidase, 0·0015 M), and were centrifuged for 20 min at 100,000 g in a refrigerated Spinco preparative ultracentrifuge. No attempt was made to separate cell debris, since earlier work had shown that less than 5 per cent of the total 5-HT was found in this fraction. Also, the earlier work had emphasized the need for rapid separation, because (1) granules isolated from brain lose a large portion of their 5-HT upon re-suspension and re-centrifugation; and (2) there is a certain affinity of particulate material of brain for 5-HT added to the suspending medium.

Immediately upon completion of the centrifugation, a 5-ml aliquot of the supernatant fraction and the residue (resuspended in 1 ml of distilled water) were individually treated with 20 volumes of absolute acetone, following the method of Amin *et al.*[4] Because of the large quantity of sucrose in the supernatant fraction, filtration had to be done under vacuum. This resulted in an opalescent acetone extract, which was concentrated *in vacuo* at 37 °C. The filtered acetone extract of the particulate fraction was similarly concentrated *in vacuo* to the aqueous phase. These were then defatted for 5 min with 10 ml of petroleum ether, and finally taken to dryness. The dried residues were stored at — 16 °C until assayed for 5-HT (within 72 hr) on the heart of the clam, *Mercenaria* (*Venus*) *mercenaria*.

The drugs used in this study were administered in doses previously shown to produce marked behavioral changes or to affect the metabolism of 5-HT. The animals were usually sacrificed after maximum effects of the drugs under study had developed. In some instances (e.g. with phenobarbital), doses were just sub-lethal. Each experiment was carried out with its own unmedicated controls in order to take into account daily fluctuating variables.

Significance of the differences of the experimental data from the control values at or beyond the 5 per cent level of confidence was calculated by the Cochran–Cox method of testing the significance of the hypothesis of equality of means, weighted in terms of their respective variances.[5] This method is used to test differences between means when variances are unequal or unknown. *P* values have been calculated from Student's "*t*" table, based on size of samples. All of the data presented below are compared for convenience with mean values from sixty-two pooled control animals. It should be pointed out that in each individual experiment the direction of change in the proportion of free 5-HT in the treated group from its control, run simultaneously, was always the same as the direction of change from the pooled control value. In Table 3 the experimental data from the preceding tables are recalculated in terms of their own individual controls and statistical significance estimated by the method described by "Student" and Fisher.[6] In Table 3, changes in distribution are expressed in terms of the percentage change in the fraction of 5-HT in the free state.

RESULTS AND DISCUSSION

The following classification is for convenience of presentation and discussion only and is made with the full realization of the complex action of these drugs on the CNS.

1. *General depressants of the CNS*

The drugs used in this category have previously been shown to raise, lower, or have no effect on the total level of 5-HT in the whole brain of the rat, while producing generally a reduction in motor activity, and, in some cases, sleep. Methylparafynol was included as an example of a drug which markedly alters cerebral acetylcholine levels.[7] Table 1 summarizes the results.

TABLE 1. INFLUENCE OF VARIOUS CNS DEPRESSANTS ON THE SUBCELLULAR DISTRIBUTION OF 5-HT

Treatment	Number of replicates	Mean 5-HT (μg/whole brain)			
		Partic. ("bound")	Supernatant ("free")	Total	% "free"
1. None	62	0·39 ± 0·09†	0·16 ± 0·04†	0·55	29
2. Chlorpromazine (25 mg/kg—75 min)	12	0·30 ($p < 0·05$)*	0·25 ($p < 0·001$)	0·55	45 ($p < 0·01$)
3. Reserpine (5 mg/kg—4 hr)	14	0·09 ($p < 0·001$)	0·06 ($p < 0·001$)	0·15	40 ($p < 0·001$)
4. α-Methyl-DOPA (100 mg/kg—1 hr)	12	0·27 ($p < 0·001$)	0·18 (n. s.)	0·45	40 ($p < 0·001$)
5. Phenobarbital (100 mg/kg—2·5 hr)	5	0·45 ($p < 0·01$)	0·33 ($p < 0·01$)	0·78	42 ($p < 0·001$)
6. Methylparafynol (300 mg/kg—15 min)	7	0·32 (n. s.)	0·14 (n. s.)	0·46	30 (n. s.)

* The p values listed were derived from Student's "t" table.
† Standard deviation.

It may be seen (as in the earlier report[1]) that 4 hr after a large dose of reserpine, the proportion of total 5-HT existing in the "free" state is considerably increased, even though the absolute amount is reduced to approximately one-third of its normal value. This is in agreement with the concept that reserpine impairs the ability of cells to store 5-HT. Because reserpine depleted the particulate fraction of about 77 per cent of the 5-HT normally found there, its net effect was to increase the proportion in the free form by about 40 per cent (see Table 3). A similar increase in free 5-HT was seen after α-methyl DOPA (reported to have a depressant action similar to that of reserpine[8]), chlorpromazine, and phenobarbital. It is of interest that these drugs are considered to be depressants of the CNS and that they produced similar net shifts in the proportion of free 5-HT regardless of the direction of change in the total 5-HT (lowered by reserpine, 72 per cent; lowered by α-methyl DOPA, 21 per cent; raised by phenobarbital, 40 per cent; and not changed by chlorpromazine).

It should be noted that isoreserpine (not shown in the table), which has weaker depressant actions than reserpine, produced changes similar to those elicited by reserpine. The results with methylparafynol are also of interest in that this drug, in a dose which produces an anesthetic state, caused little change in the subcellular distribution of 5-HT; on the other hand, it is worth pointing out that this agent has been shown to influence the total level of acetylcholine in the brain of the rat.[7]

It may be observed in Table 3 that the same order and direction of effect is found whether one compares the results with the pooled controls or with the appropriate internal and simultaneous controls. Table 3 also shows that among the depressants

tested (group I) all except methylparafynol produced statistically significant increases in the proportion of free 5-HT.

2. *Anti-Depressants and LSD-25*

In order to study a contrasting situation, a group of agents which produce effects on the CNS essentially opposed to those elicited by the group in (1) above, were investigated for their influence on the intracellular distribution of 5-HT. A summary of these data appear in Table 2.

TABLE 2. INFLUENCE OF CERTAIN ANTI-DEPRESSANTS AND A PSYCHOTOMIMETIC AGENT ON THE SUBCELLULAR DSTRIBUTION OF 5-HT

Treatment	Number of replicates	Mean 5-HT (μg/whole brain)			
		Partic. ("bound")	Supernate ("free")	Total	% "free"
1. Iproniazid (100 mg/kg—12 hr)	17	1·00 ($p < 0·001$)*	0·49 ($p < 0·001$)	1·49	33 (n. s.)
2. β-Phenyl*iso*propyl-hydrazine (10 mg/kg—18 hr)	16	1·05 ($p < 0·001$)	0·34 ($p < 0·001$)	1·39	24 (n. s.)
3. Imipramine (15 mg/kg—75 min)	16	0·46 ($p < 0·01$)	0·17 (n. s.)	0·63	27 (n. s.)
4. LSD-25 (1300 μg/kg—20 min)	8	0·53 ($p < 0·01$)	0·16 (n. s.)	0·69	23 ($p < 0·01$)
5. None	62	0·39	0·16	0·55	29

* The p values listed were derived from Student's "t" table.

TABLE 3. INFLUENCE OF VARIOUS DRUGS ON THE SUBCELLULAR DISTRIBUTION OF 5-HT DETERMINED FROM SIMULTANEOUS CONTROLS

Drug	Number of replicates	Fraction of total 5-HT in free form		% Change from control value†	P*
		Treated	Control		
Group I					
1. Chlorpromazine	12	0·45	0·33	− 36	< 0·02
2. Reserpine	12	0·41	0·29	− 41	< 0·001
3. α-Methyl DOPA	12	0·41	0·33	+ 24	< 0·03
4. Phenobarbital	5	0·42	0·30	+ 40	< 0·001
5. Methylparafynol	7	0·30	0·27	+ 2	n. s.
Group II					
1. Iproniazid	17	0·35	0·33	+ 6	n. s.
2. β-Phenyl*iso*propyl-hydrazine	16	0·25	0·29	− 14	< 0·05
3. Imipramine	16	0·27	0·32	− 16	< 0·05
4. LSD-25	14	0·23	0·28	− 18	< 0·001

* Determined by "t" test of Student.

† Calculated by the following formula: $\frac{\text{treated} - \text{control}}{\text{control}} \times 100.$

The results obtained with the agents shown in this table seem to fit a trend opposite to that seen in Table 1. Of the compounds in Table 2, none of which produces a reduction in spontaneous motor activity, all except LSD-25 elicit a rise in total 5-HT and no significant change in the proportion of free 5-HT. The most striking result was that obtained with LSD-25. At a time when the dose of LSD-25 had exerted a marked effect on the conditioned behavior of rats,[9] the level of total 5-HT in the brain was increased by 25 per cent, all of which could be accounted for in the particulate fraction. Thus, LSD-25 appears to cause an increased binding and storage of 5-HT.[10]

The dose of LSD-25 used in this study (1300 μg/kg) is extremely high relative to an hallucinogenic dose in man. However, similar changes in subcellular 5-HT and elevations of total 5-HT have been found with doses of 130 μg/kg.[10, 11] It is interesting that this is the threshold dose in the rat for LSD-25 to elicit both impairment of behavior in fixed-ratio and other operant schedules and autonomic disturbances.[9] It is also of interest that the greatest LSD-25-induced elevation in cerebral 5-HT occurs in from 20 to 30 min, at the peak of the behavioral effect of the drug, and that the 5-HT levels return to normal at 4 hr, when the behavioral disturbance has subsided.[10]

Another interesting aspect of the data shown in Table 2 is that the increase in the levels of total 5-HT in the brain following inhibition of monoamine oxidase (MAO) by iproniazid was distributed between the particulate and non-particulate compartments with no significant change in distribution (cf. Table 3). These results suggest the existence of a dynamic steady-state relationship between the concentration of 5-HT in the stored reserves and that free in the cytoplasm. Presumably, this relationship would be maintained normally by a balance between synthesis and storage of the amine, and the rates of release and metabolism of the free amine.

Table 3 again shows that the direction and magnitude of change produced by the drugs in group II are the same whether measured from the pooled control group or from the internal simultaneous controls. Statistically, the change in distribution following iproniazid was not significant, while those following β-phenyl*iso*propylhydrazine and imipramine were of border-line significance, and that following LSD was highly significant.

3. *Interaction with reserpine*

In an earlier work, Giarman and Schanberg[1] showed that iproniazid interferes with reserpine-induced release of 5-HT from brain particles. This is in agreement with the work of Green and Sawyer,[12] which also demonstrated a similar effect of another inhibitor of MAO, i.e. tranylcypromine, on reserpine-induced release of granule-bound catecholamines in rat brain. Furthermore, Pepeu *et al.*[13] have shown a differential action of iproniazid and β-phenyl*iso*propylhydrazine (PIH) on spontaneous release of catecholamines from isolated atria of the guinea pig, regardless of complete inhibition of MAO. Consequently these compounds were tested in this system for their ability to resist reserpine-induced release of 5-HT. The results of these experiments are summarized in Table 4.

It is clear from these data that although iproniazid, PIH, impramine and 5-HTP all increase the level of 5-HT in the brain of the intact rat, only iproniazid both

completely inhibits the releasing action of reserpine and maintains the granule-bound concentration of 5-HT at a level equivalent to that observed following the administration of iproniazid alone. PIH was much less potent in this anti-reserpine effect, reducing the reserpine-depleting action on the particulate fraction from an uninhibited 77 per cent (0·39 μg 5-HT to 0·09) to 37 per cent (1·05 μg 5-HT to 0·67). Since PIH

TABLE 4. EFFECT OF MAO-INHIBITORS, IMIPRAMINE, AND 5-HYDROXYTRYPTOPHAN ON RESERPINE-INDUCED RELEASE OF 5-HT FROM BRAIN PARTICLES

Treatment	Number of animals	Mean 5-HT (μg/whole brain)			
		Partic. ("bound")	Supernate ("free")	Total	% "free"
1. None	62	0·39	0·16	0·55	29
2. Reserpine (5 mg/kg—4 hr)	14	0·09	0·06	0·15	40
3. Iproniazid (100 mg/kg—12 hr)	17	1·00	0·49	1·49	33
4. Iproniazid (3) followed by reserpine (2)	16	1·00	0·54	1·54	35
5. PIH* (10 mg/kg—18 hr)	16	1·05	0·34	1·39	24
6. PIH (5) followed by reserpine (2)	20	0·67	0·34	1·01	34
7. 5-Hydroxytryptophan (100 mg/kg—30 min)	12	0·79	0·67	1·46	46
8. 5-Hydroxytryptophan (7) followed by reserpine (2)	10	0·35	0·30	0·65	46
9. Imipramine (15 mg/kg—75 min)	4	0·51	0·20	0·71	27
10. Imipramine (9) followed by reserpine (2)	12	0·07	0·03	0·10	30

* β-Phenyl*iso*propylhydrazine.

was used here in a dose roughly equivalent in MAO-inhibiting potency to that of iproniazid, the anti-reserpine effect cannot be attributed solely to the action of iproniazid on MAO, nor to the high initial levels of 5-HT upon which the reserpine must act, because in the presence of equally high levels of 5-HT induced by 5-HTP, reserpine was still able to reduce granule-bound 5-HT by 56 per cent (0·79 μg 5-HT to 0·35). Thus, it would appear that iproniazid exerts some action (apart from its inhibition of MAO) on the 5-HT storage-granule, rendering it insusceptible to the amine-releasing action of reserpine. These findings are in support of Pletscher's suggestion[14] that iproniazid inhibits the 5-HT-releasing action of reserpine, and are in disagreement with Brodie's contention[15] that iproniazid does not affect the release of 5-HT from platelets or other organs.

GENERAL DISCUSSION

The results of this investigation support the view that 5-HT in brain is present in two separable forms: (1) that associated with the particulate material; and (2) that present in particulate-free cytoplasm. The demonstration that some neuropharma-

cologic agents can elicit a shift in levels of "bound" and "free" amine, thus altering the normal equilibrium of distribution of 5-HT within the cell, lends credence to the general theory that the action of some drugs may be correlated with changes in the internal distribution of 5-HT in the brain. It is of great interest that a depressant of the CNS, such as phenobarbital, which leads to an increase in the level of total 5-HT in brain, is also associated with an increase in the percentage of free 5-HT, similar to that seen with reserpine and α-methyl-DOPA, which lower total cerebral 5-HT, and with chlorpromazine which does not change total 5-HT. These results with chlorpromazine seem to fit well the suggestion of Gey and Pletscher that this drug acts to change the permeability of the storage organelles for the monoamines.[15] Such an action might indeed extend to other drugs in this series. It would seem, therefore, that the subcellular distribution of 5-HT may be a more reliable correlate of the effect of a particular drug on the overall activity of the CNS than the total 5-HT level in the brain.

A working generalization arising from these studies might be stated as follows: (*a*) irrespective of the change induced in the total level of 5-HT in the brain, some depressants of the CNS are likely to cause an increase in the proportionate amount of free 5-HT; and (*b*) some drugs which are anti-depressants, euphoriants, mild stimulants or hallucinogenic are likely to cause an increase in the total level of 5-HT, while decreasing or leaving unchanged the percentage of free 5-HT.

This statement is not meant to imply that the subcellular distribution of 5-HT can be predicted *a priori* on the basis of the behavioral changes evoked. Methylparafynol, a depressant, and the 3-methyl derivative of pentobarbital, a convulsant, for example, had no effect upon the subcellular distribution of 5-HT in brain.

At the present time these data on drug-induced changes in the subcellular distribution of 5-HT must be viewed only as correlations with drug-induced behavioral changes with no suggestion of a causal relationship. Such correlations, however, may be indicative of underlying mechanisms. It should be emphasized that there is no evidence as yet that 5-HT acts primarily as a transmitter. It is possible that this and other neurohumoral amines may function as neuroregulatory substances by modifying the environment in which transmitters act. Ordinarily, the "free" form is considered to be the active form. It is our view, however, that the "free" 5-HT measured in the supernatant fraction in this work is a transition form, potentially active, potentially storable, and potentially destructible. In this same view, the particulate fraction may be an estimate of the stored neurohumor (granule-bound) and the active neurohumor (complexed with specific receptors leading to the evoked response). Thus, the neuroregulatory action of 5-HT probably depends more upon the relative concentration-distribution among these three forms than upon any single level or form.

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Note added in proof: Since the preparation and submission of this report, a number of these experiments have been repeated, under the same conditions, with the exception that the 5-HT was extracted by the alkaline–butanol method and estimated spectrophotofluorometrically. Although the absolute levels of 5-HT obtained with this procedure were somewhat higher than those described in the paper, the magnitude and direction of change produced in both subcellular fractions by LSD-25, phenobarbital and reserpine were the same as those reported herein. The other drugs were not re-tested.