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# VESICULAR AND JUXTAVESICULAR SEROTONIN: EFFECTS OF LYSERGIC ACID DIETHYLAMIDE AND RESERPINE

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## ABSTRACT

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An extensive analysis of subcellular serotonin (5-HT) compartmentation with and without reserpine was undertaken in order to localize further the effect of LSD on rat brain 5-HT. Modification of the subfractionation procedure resulted in an increase in purity of fractions and a decrease in variability of 5-HT content. With the modified procedure, the administration of LSD produced a significant increase in 5-HT in the nerve-ending fraction prepared from rat whole brain. LSD caused a 50% increase in 5-HT in the vesicular fraction which was recovered after osmotic disruption of nerve-endings. The increase of 5-HT in the vesicular fraction after LSD was not demonstrable in rats treated with reserpine for as long as 2 weeks postreserpine. Instead, with reserpine pretreatment the LSD-induced increase in 5-HT was localized to the intrasynaptosomally derived "end supernatant" as early as 48 hours postreserpine. Thus, an unanticipated "juxtavesicular" site capable of 5-HT retention or binding was detected. In crude subcellular fractions, by contrast, significant increases in 5-HT were not observed with LSD administration until 4 days after reserpine, at which time at least a 50% 5-HT repletion had occurred. This study of drug interactions suggests a juxtavesicular compartment that may be of functional importance in presynaptic binding or transport of 5-HT.

The well documented effect of *d*-lysergic acid diethylamide (LSD) on rat brain serotonin (5-HT) metabolism entails an increase of 5-HT within the first 60 minutes after drug administration and a rapid, early decrease of 5-hydroxyindoleacetic acid (5-HIAA) (Rosecrans *et al.*, 1967; Freedman *et al.*, 1970; Freedman and Boggan, 1974; Diaz *et al.*, 1968; Andén *et al.*, 1971). The decrease in 5-HIAA represents a largely unexplained inaccessibility of the substrate to monoamine oxidase (MAO), since efforts to detect an inhibitory effect of LSD on MAO have been unsuccessful (Freedman, 1961a,b; Collins *et al.*, 1970). The subcellular localization of the LSD-induced increase in 5-HT, initially described as enhanced binding of substrate, has been partially described, but the source and mechanism of the increase in brain 5-HT after LSD has not been fully explained. The feedback-mediated reduction in conversion of labeled tryptophan to 5-HT after LSD (Lin *et al.*, 1969; Schubert *et al.*, 1970; Shields and Eccleston, 1973) is relevant to the later effects of LSD on indoleamine metabolism rather than to the initial increase in 5-HT and decrease in

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5-HIAA. To account for these early effects, the action of LSD on the raphe soma, causing cessation of firing (Aghajanian *et al.*, 1970, 1972), and local membrane effects at the nerve-ending which might account for inhibition of release (Chase *et al.*, 1967) along with enhanced binding of 5-HT (Halaris *et al.*, 1972; Halaris and Lovell, 1973) have been postulated as component mechanisms.

Whatever the mechanism, the increase in 5-HT after LSD was localized almost entirely in a high speed particulate fraction (Freedman, 1961a,b), an effect unique to LSD (Schanberg and Giarman, 1962; Giarman *et al.*, 1964). An attempt to localize further the subcellular site of the increase in 5-HT after LSD (Rosecrans *et al.*, 1967) indicated that all particulate fractions tested contained variable and small increases of 5-HT after LSD. In the initial description of an effect of LSD on brain 5-HT, Freedman (1961a) concluded that LSD induced a binding of 5-HT within a storage site and stimulated the recovery of binding capacity after pretreatment with reserpine. Since early effects of LSD are somehow linked with amine storage and/or release, we undertook a more extensive analysis of subcellular compartmentation of 5-HT to localize further the effect of LSD alone and after reserpine. The results clearly demonstrate that the predominant site of the LSD-induced enhanced binding or retention of 5-HT is the synaptic vesicle. After impairment of this storage site with reserpine, a new intrasynaptosomally derived soluble storage or binding site shows enhanced binding of 5-HT after LSD.

## Methods

Male Sprague-Dawley rats (Madison, Wisc.), 200–300 g in weight, were used. Animals were kept in an air-conditioned room at 22°C and 55% relative humidity with a 7 A.M. to 7 P.M. light-dark cycle. The rats were housed six per cage and had *ad libitum* access to Purina rat chow and water.

Reserpine (Serpasil, Ciba Pharmaceutical Company, Summit, N. J.) was injected i.p. at a dose of 5 mg/kg. Control animals were injected with placebo as obtained from Ciba Pharmaceutical Company in equivalent volumes. Animals were sacrificed in the A.M. by decapitation at various time intervals ranging from 12 hours to 15 days after reserpine. The body weight was always measured at 24 hours after reserpine or, for earlier intervals, before sacrifice. Animals that lost less than 5% body weight after reserpine were excluded (Halaris and Freedman, 1975). LSD [5-R, 8-R(+)-LSD tartrate] (520 μg/kg i.p.) was given in a volume of 2.5 ml/kg and animals were sacrificed at intervals ranging from 15 to 60 minutes.

**Subcellular fractionation.** A modified method of Eichberg *et al.* (1964) was used. Two rat brains were pooled and homogenized (12 strokes, 1 minute) in 10 volumes of ice-cold 0.32 M sucrose (containing 60 μg of the monoamine oxidase inhibitor *p*-tolyl ether of choline per ml) in Tri-R glass homogenizers with Teflon pestles (clearance 0.009–0.011 inch). The homogenate was spun at 1000 × *g* for 10 minutes in a Sorvall RC 2-B refrigerated centrifuge. The supernatant ($P_1S$) was carefully separated from the pellet ($P_1$) and the pellet was washed once with 5 ml of 0.32 M sucrose. The wash was added to $P_1S$, and the mixture was centrifuged at 12,000 × *g* for 20 minutes in the Sorvall to give the crude mitochondrial pellet ($P_2$) and a supernatant ($P_2S$). The latter was spun at 200,000 × *g* for 30 minutes in the International B-60 ultracentrifuge to obtain the cellular microsomal fraction ($P_3$) and the true supernatant ($P_3S$). The $P_2$ pellet was subfractionated into light myelin ($P_2A$), nerve-endings ($P_2B$) and purified mitochondria ($P_2C$) by using a discontinuous sucrose density-gradient as originally described by Gray and Whittaker (1962) except that the gradient was spun at 100,000 × *g* for 30 minutes (instead of 53,500 × *g* for 120 minutes) in the swing-out SB-110 rotor of the International B-60 ultracentrifuge.

After aspiration of the bands, the $P_2B$ suspension was diluted with an equal volume of ice-cold deionized water and centrifuged at 200,000 × *g* for 30 minutes to give a well packed pellet. The $P_2B$ pellet was disrupted by osmotic shock as described by Whittaker *et al.* (1964). Ice-cold deionized water (2 ml/g of original tissue) was added and the pellet was dissolved by means of a Sonifier model W185 (Heat Systems-Ultrasonics, Inc. Plainview, Long Island, N.Y.) with 40 w for 45 seconds. The water suspension was centrifuged at 12,000 × *g* for 20 minutes resulting in a cloudy supernatant and a loosely packed pellet (Wp). This supernatant was spun at 200,000 × *g* for 30 minutes to give a pellet rich in synaptic vesicles ($P_2V$) and a clear "end supernatant" (Se).

**Amine assays.** Serotonin was determined essentially according to the method of Andén and Magnusson (1967) and 5-HIAA by the method of Jonsson and Lewander (1970). Serotonin was extracted from pellets after resuspension of the pellet in 0.32 M sucrose and addition of 4 N perchloric acid to make a final concentration of 0.4 N perchloric acid. Supernatants were treated in a similar manner. Serotonin from subcellular fractions was reacted with O-phthalaldehyde reagent as described by Maickel *et al.* (1968). Standards were run with each assay and the values were corrected for recovery. The activation and emission spectra were checked for each individual sample.

**Protein.** The protein content of pellet suspensions

or supernatants was determined by the method of Lowry *et al.* (1951) using appropriate aliquots.

**Electron microscopy.** Two independent preparations of each fraction were examined. Fractions were centrifuged at 100,000 × *g* for 30 minutes to form a firm pellet. The pellet was fixed for 2 hours in phosphate-buffered 5% glutaraldehyde (pH 7.4) and after a wash in phosphate buffer it was postfixed for 1 hour with 1% osmium tetroxide. The pellet was then dehydrated in graded ethanols and embedded in Epon. Sections were cut in a Sorvall MT 2 ultramicrotome, double-stained with uranyl acetate and lead hydroxide and examined in a RCA 4 electron microscope.

**Choline acetyltransferase (ChAT).** Enzyme activity was determined in $P_2B$ and $P_2V$ fractions as well as in the Se. The procedure of Fonnum (1975) was followed. A boiled tissue blank for each fraction was included. The radioactivity accumulated in the tissue blank was assumed to be nonspecific and was subtracted from that of the sample. Radioactivity was counted in an Isocap 300 (Searle Radiographics, Inc., Chicago, Ill.) liquid scintillation spectrometer. Three independent determinations of each fraction were performed.

## Results

**Modified subfractionation.** The major modifications were the substantial shortening of the spinning time (30 *vs.* 120 minutes) and the increase in centrifugal force (100,000 *vs.* 53,500 × *g*) utilized to subfractionate the $P_2$. These changes significantly enhanced the compactness and distinctiveness of the bands and they visibly increased the clarity of the phases. Aspiration of the bands from the sucrose phases of the gradient was thus greatly facilitated. The modification of the procedure resulted in reduced variability, a satisfactory consistency in control values of 5-HT and a higher protein content of the $P_2A$ and the $P_2B$ fractions. After the long procedure, the average protein content of these two fractions was 4 and 10 mg/g of brain, respectively, *vs.* 11 and 14 mg/g of brain with the short spin ($P < .05$). The protein content of the $P_2C$ remained unchanged (4 mg/g of brain). The higher protein content of $P_2A$ and $P_2B$ after the shorter spin is probably related to the enhanced clarity of the phases of the gradient. Finally, the concentration of 5-HT (expressed as nanograms per milligram of protein) was virtually unaffected whether the long or short procedure was used although mitochondrial 5-HT was slightly higher after the short spin. The modification was thus useful, although not indispensable, in measurements of changes of small magnitude occurring in fractions with low amine content. Its practical advantage is to accomplish the entire subfractionation and amine measures within 8 to 10 hours rather than the usual 12 to 14 hours.

Electron micrographs of fractions $P_2A$, $P_2B$ and $P_2C$ were basically in accord with the results of Gray and Whittaker (1962) (fig. 1). The abbreviated procedure resulted in slightly better preservation of nerve-endings. Fraction $P_2A$ (not shown) consisted of myelin fragments with an occasional undisrupted axon. About 80% of the particles in fraction $P_2B$ (fig. 1A) were large and small nerve-endings. There was a 15 to 20% mitochondrial contamination as well as occasional microsomes and very rarely a myelin fragment. Fraction $P_2C$ (fig. 1B) was a fairly pure mitochondrial fraction with large synaptosomes not exceeding 15% of the population. The combined use of ultrasound and osmotic shock resulted in a more efficient disruption of nerve-endings than that described by Whittaker *et al.* (1964). The debris fraction (Wp) (fig. 1C) consisted mainly of large mitochondria, myelin fragments and a considerable number of partially disrupted synaptosomes. The structure of these elements was poorly preserved. Fraction $P_2V$ (fig. 1D) was rich in hollow, dense-core and compound synaptic vesicles, the structural integrity of which was largely unaffected. There was a fair number of nonvesicular membrane fragments probably of microsomal origin. Thick nonvesicular membranes probably of postsynaptic origin could also be detected. Very small undisrupted nerve-endings could be seen occasionally but there was virtual absence of mitochondria.

Table 1 shows the presence of ChAT activity in relevant subcellular fractions. Specific enzyme activity in the vesicular fraction is about twice that found in the purified nerve-ending fraction. The end supernatant contains about one-third of the activity recovered in vesicles and this activity is probably due to soluble enzyme. Finally, purified mitochondria contain traces of ChAT activity due to contaminating nerve-endings also revealed by the electron microscope.

**Subcellular 5-HT after LSD.** Significant 5-HT increases after one i.p. dose of LSD (520 μg/kg) were obtained only in the crude mitochondrial and synaptosomal fractions (table 2). This increase was 29 and 25% respectively. The increase in synaptosomal 5-HT was not only

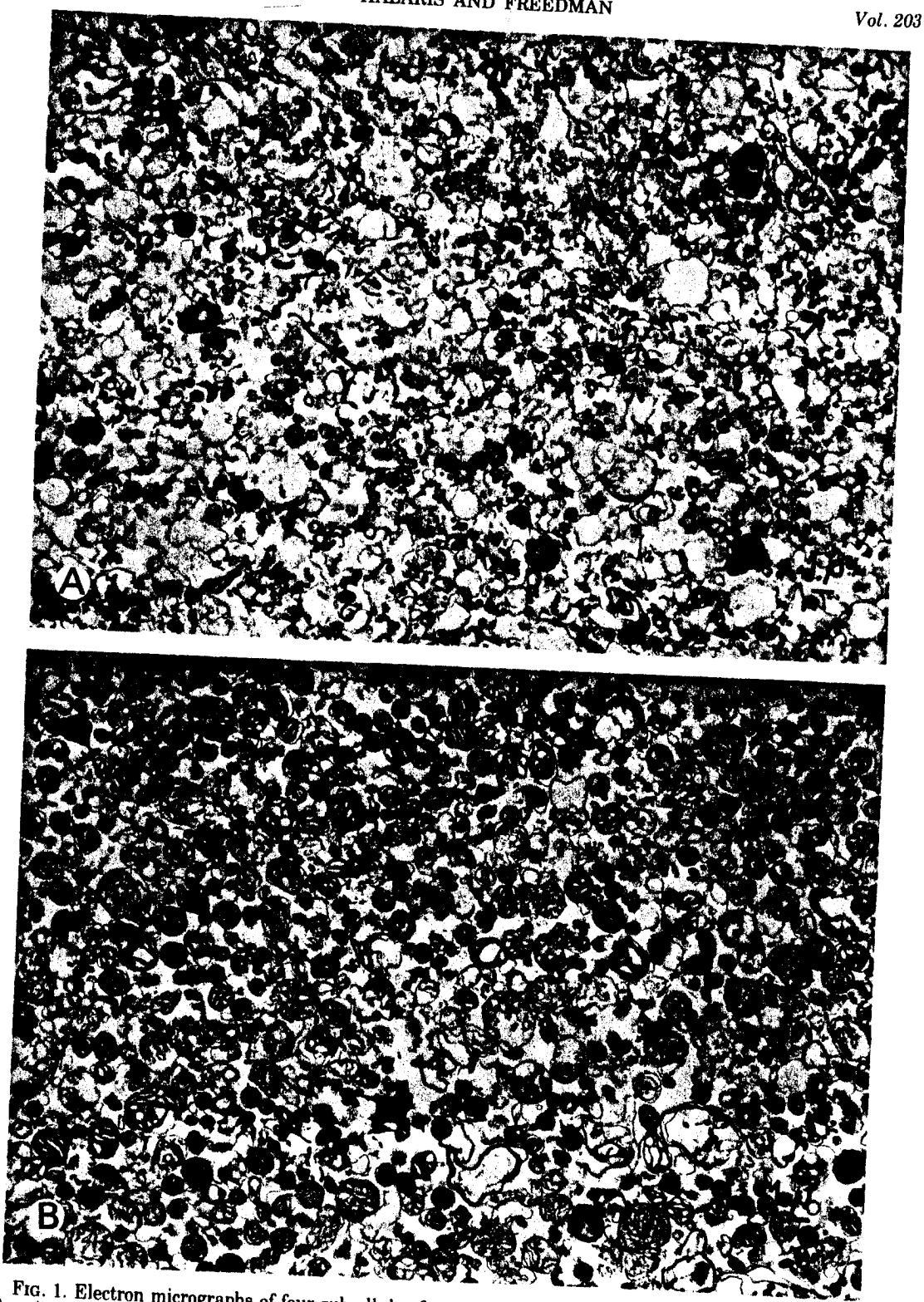
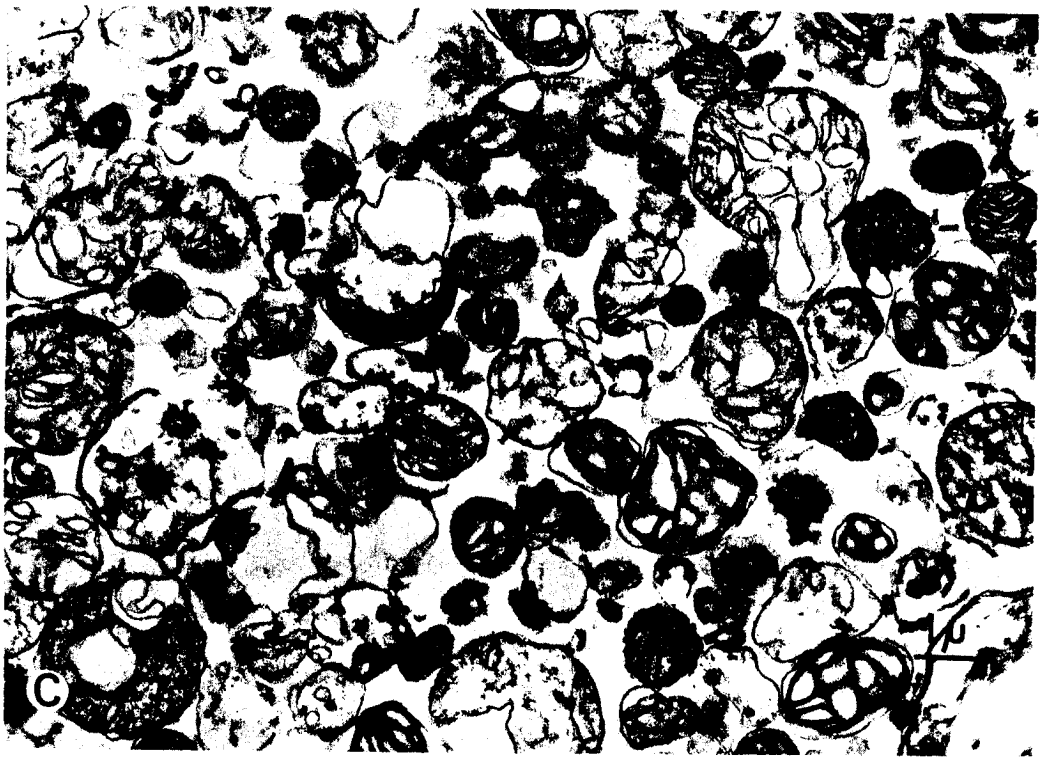



FIG. 1. Electron micrographs of four subcellular fractions. A. $P_2B$; B. $P_2C$; C. Wp; D. $P_2V$. See text for description of fractions. Magnification is indicated by the marker line.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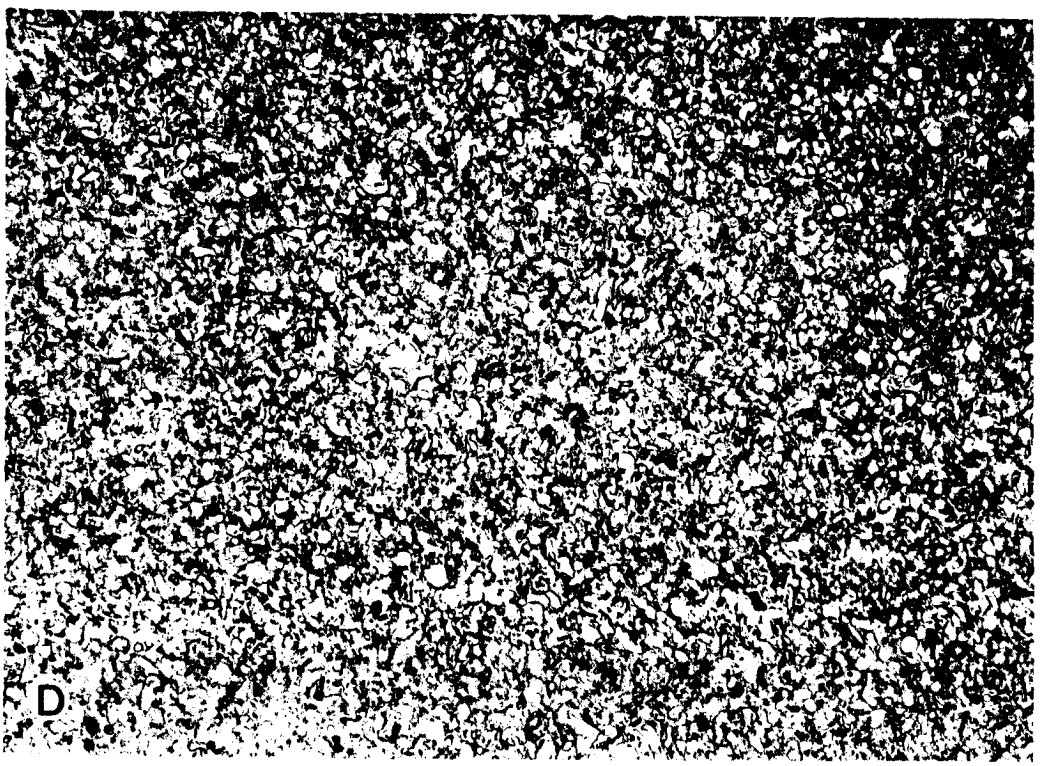
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reliable and consistently reproducible, but showed no overlap with control values as was the case with the variable 5-HT increase in mitochondria and microsomes. The true supernatant consistently showed a small (12%) decrease which was not statistically significant. This decrease is noteworthy because 5-HT in the supernatants has always been observed to decrease after an acute dose of LSD.

In the $P_2V$, measured after disruption of the nerve-endings by combined osmotic shock and ultrasound, the LSD-induced increase in 5-HT was 50% (fig. 2), the largest observed in any of the fractions. This increase was statistically highly significant ($P < .005$). There was a slight decrease (5%) of 5-HT in the Se (fig. 2), the fraction that showed the significant 5-HT increase after LSD in reserpine-treated animals (*vide infra*).

**Effect of LSD on whole brain 5-HT after reserpine.** At 12 and 24 hours after reserpine, animals were administered LSD (520 μg/kg) and killed 15, 30, 45 and 60 minutes later. Reserpine alone caused significant 5-HT depletion that had already reached a maximum by 12 hours. There was no significant effect of LSD on whole brain 5-HT at any of these time intervals (fig. 3). At later times after reserpine

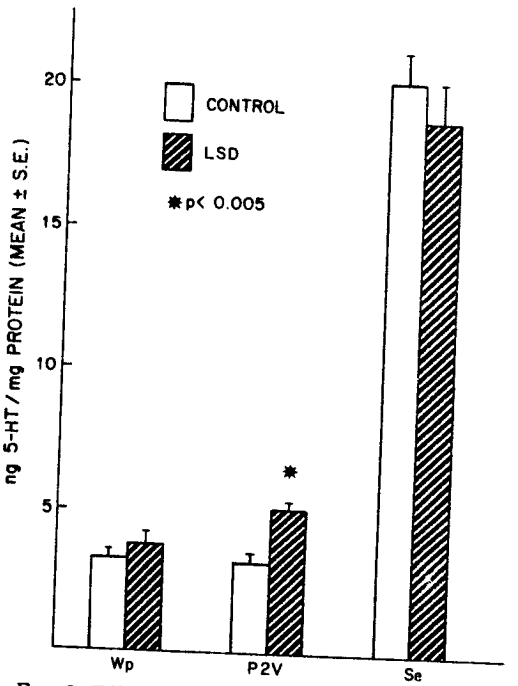


FIG. 2. Effect of LSD (520 μg/kg i.p.) on 5-HT of synaptosomal subfractions after disruption of the nerve-endings by combined osmotic shock and ultrasound as described under "Methods." Animals (eight in each group) were sacrificed 45 minutes after the drug; control animals were injected with saline. Values are the mean ± S.E.M. nanograms of 5-HT per milligram of protein. Total protein content was 20 mg in the Wp, 22 mg in the $P_2V$ and 12 mg in the Se. $P < .005$ by the two-tailed Student's *t* test.

TABLE 1

*ChAT activity in subcellular fractions*

| Tissue Fraction | Specific Enzyme Activity[a] | Total Enzyme Activity |
|---|---|---|
| Nerve-endings | 4.8 | 192.0 |
| Synaptic vesicles | 9.1 | 200.2 |
| End supernatant | 3.0 | 36.0 |
| Purified mitochondria | 1.0 | 12.5 |

[a] Specific activity is expressed in micromoles per hour per milligram of protein.

TABLE 2

*Effect of LSD on the subcellular localization of brain 5-HT[a]*

| Tissue Fraction | Control Animals[b] | LSD-treated Animals[b] | % of Control |
|---|---|---|---|
| | *ng/mg protein* | | |
| Crude mitochondria | 7.3 ± 0.3 (4) | 9.4 ± 0.3[c] (4) | 129 |
| Light myelin | 2.6 ± 0.3 (9) | 3.0 ± 0.7 (9) | 115 |
| Nerve-endings | 6.0 ± 0.3 (9) | 7.5 ± 0.4[d] (9) | 125 |
| Mitochondria | 3.5 ± 0.7 (8) | 3.8 ± 0.6 (8) | 108 |
| Microsomes | 8.0 ± 0.6 (9) | 9.0 ± 0.6 (9) | 113 |
| True supernatant | 4.3 ± 0.1 (9) | 3.8 ± 0.6 (9) | 88 |

[a] Rats were given an i.p. injection of LSD (520 μg/kg) and sacrificed 45 minutes later.

[b] Each value represents the mean 5-HT level ± S.E. for the number of animals in parentheses. Total protein content was 86 mg in the crude mitochondrial fraction, 34 mg in the light myelin fraction, 42 mg in the nerve-endings, 12 mg in pure mitochondria, 35 mg in the microsomes and 70 mg in the true supernatant.

[c] $P < .005$.

[d] $P < .05$.

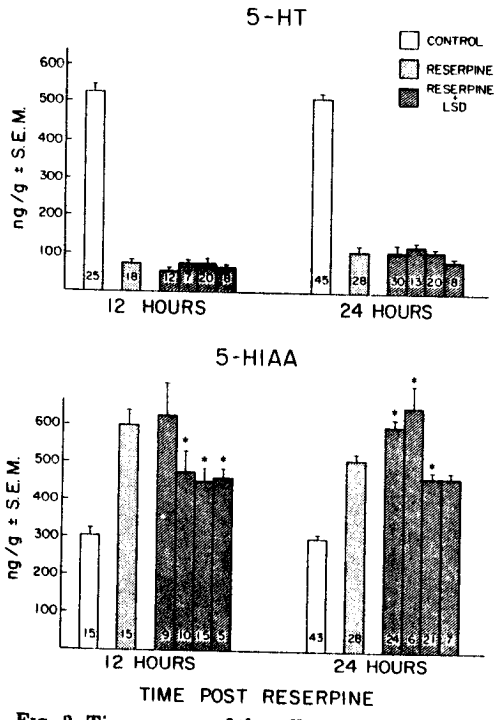


FIG. 3. Time course of the effects of LSD on brain 5-HT and 5-HIAA at early intervals (12 and 24 hours) after reserpine (5 mg/kg i.p.). Control animals were injected with reserpine-placebo and saline. Animals were sacrificed at 15, 30, 45 and 60 minutes after LSD (520 μg/kg i.p.). The hatched bars indicate these time points from left to right. The number of animals is shown inside the bars. Statistical significance with regard to animals receiving reserpine only was determined by the two-tailed Student's *t* test: *P < .05.

(48, 72 and 96 hours) the effect of LSD was assessed only at 45 minutes after the drug (fig. 4), when the expected 5-HT increase is at a peak. LSD caused an 18% increase in whole brain 5-HT at 48 hours and statistically significant increases (18–22%) at 72 and 96 hours after reserpine. At 72 and 96 hours after reserpine alone, whole brain 5-HT levels were 38 and 50% of control (levels), respectively.

**Effect of LSD on whole brain 5-HIAA after reserpine.** There was an expected increase in brain 5-HIAA after reserpine; this increase resulted in a doubling of control levels at 12 hours and gradually declined until control levels were attained at 72 hours after reserpine (fig. 4). Twelve hours after reserpine, LSD caused a significant reduction of 5-HIAA (21–25%) when animals were sacrificed 30, 45 and 60 minutes after the drug (fig. 3). Twenty-four hours after reserpine, 5-HIAA levels were increased 17 and 28% during the first 30 minutes after LSD and the significant LSD-induced decrease occurred at 45 minutes (fig. 3). Forty-eight hours after reserpine LSD had no significant effect on 5-HIAA but at both 72 and 96 hours after reserpine 5-HIAA was significantly reduced (16–18%) 45 minutes after LSD (fig. 4).

**Effect of reserpine on subcellular 5-HT.** At 12, 24, 48, 72 and 96 hours after reserpine, four crude fractions were analyzed: $P_1$, $P_2$, $P_3$ and $P_3S$. The results of this analysis are shown in figure 5.

The three particulate fractions ($P_1$, $P_2$ and $P_3$) overall showed comparable 5-HT depletion (15–18% of control levels) 12 hours after reserpine. Similarly, the repletion rate was not significantly different among these fractions. Levels of 5-HT ranged between 15 and 26% of control at 24 hours, between 24 and 33% of control at 48 hours, between 39 and 56% of

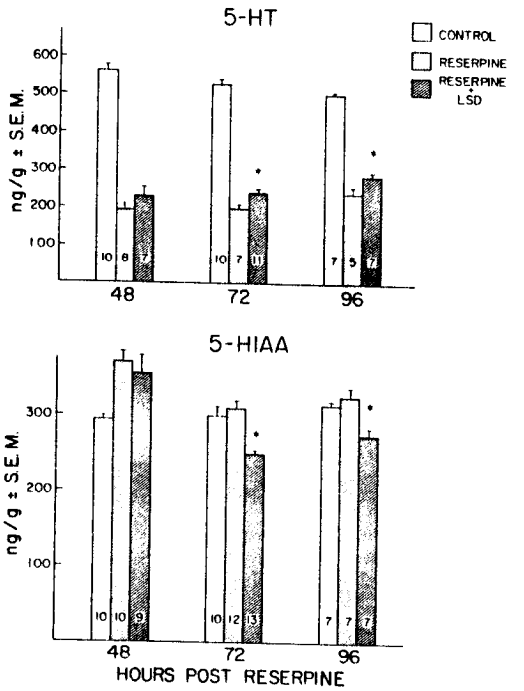


FIG. 4. Effects of LSD on brain 5-HT and 5-HIAA at later intervals (48, 72 and 96 hours) after reserpine (5 mg/kg i.p.). Control animals were injected with reserpine-placebo and saline. Experimental animals were sacrificed 45 minutes after LSD (520 μg/kg i.p.). The number of animals is shown inside the bars. Statistical significance with regard to animals receiving reserpine only was determined by the two-tailed Student's *t* test: *P < .05.

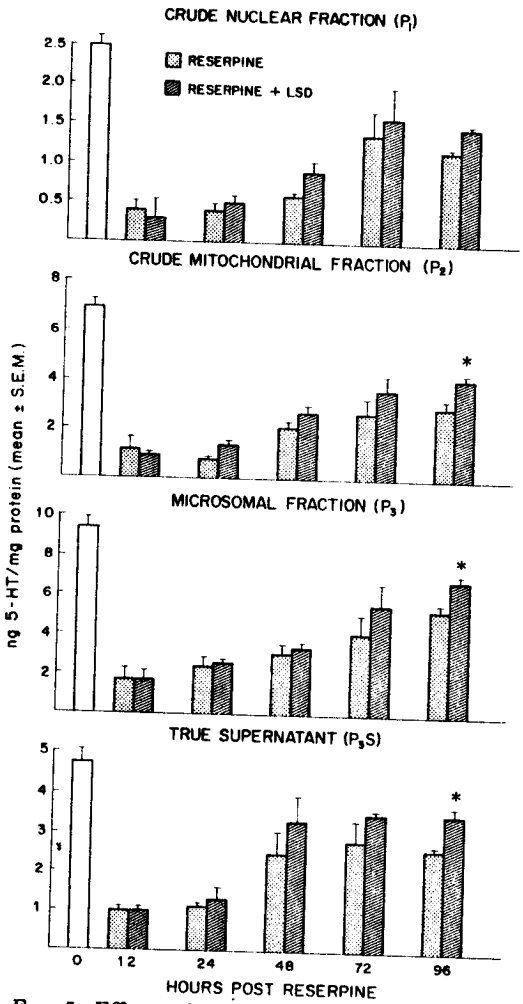


FIG. 5. Effects of LSD on the 5-HT content of crude subcellular fractions at various intervals after reserpine (5 mg/kg i.p.). Animals (four to seven in each group) were sacrificed 45 minutes after LSD (520 μg/kg i.p.); control animals (22–29 in each group) were injected with reserpine-placebo and saline. Statistical significance with regard to animals receiving reserpine only was determined by the two-tailed Student's $t$ test: *$P < .025$. Total protein content was 300 mg in the $P_1$, 86 mg in the $P_2$, 35 mg in the $P_3$ and 70 mg in the $P_3S$.

control at 72 hours and between 43 and 57% of control at 96 hours after reserpine.

In the true supernatant ($P_3S$), however, the rate of 5-HT repletion was considerably faster than in the above particulate fractions. Maximal 5-HT depletion was reached at or before 12 hours after reserpine. Levels were 21% of control at 24 hours after reserpine. A 50% repletion was reached already at 48 hours. Levels of 5-HT in this fraction were about 70% of control both at 72 and 96 hours after reserpine.

The effect of reserpine on 5-HT depletion in synaptosomal subfractions is shown in figure 6. The rate of repletion in the Wp and the $P_2V$ fractions was almost identical. The following time course was observed compared with reserpine-placebo control animals: 34 to 42% of control levels at 2 days after reserpine, 50 to 52% at 4 days, 55 to 57% at 7 days and 73 to 79% at 15 days. Repletion in the Se was similar.

**Effect of LSD on subcellular 5-HT in reserpine-treated animals.** At 12, 24, 48, 72 and 96

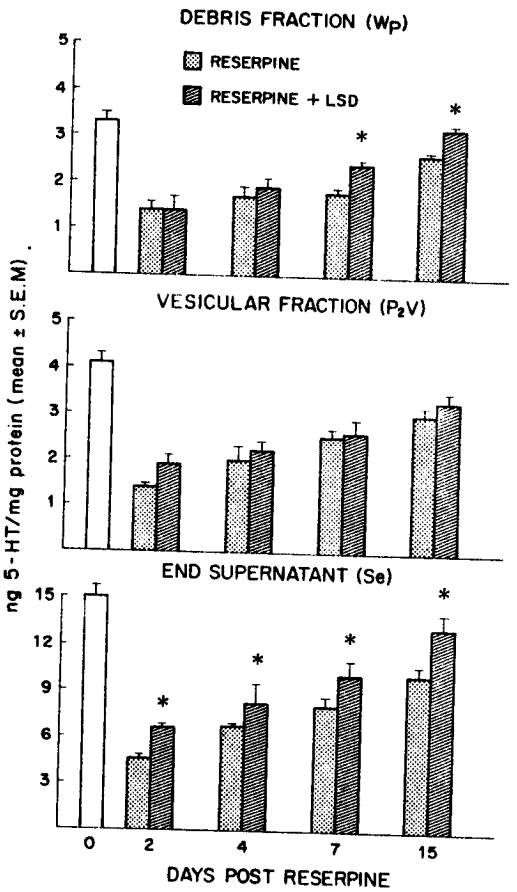


FIG. 6. Effect of LSD on the 5-HT content of synaptosomal subfractions at various intervals after reserpine (5 mg/kg i.p.). Animals (four in each group) were sacrificed 45 minutes after LSD (520 μg/kp i.p.); control animals (12 in each group) were injected with reserpine-placebo and saline. Statistical significance with regard to animals receiving reserpine only was determined by the two-tailed Student's $t$ test: *$P < .05$. Total protein content was 20 mg in the Wp, 22 mg in the $P_2V$ and 12 mg in the Se.

hours after reserpine, the effect of LSD on subcellular 5-HT was analyzed in the following crude subcellular fractions: $P_1$, $P_2$, $P_3$ and $P_3S$. Animals were sacrificed 45 minutes after LSD. The drug had no significant effect in any fraction at 12, 24, 48 and 72 hours after reserpine. Significant LSD-induced increases in subcellular 5-HT were observed in $P_2$, $P_3$ and $P_3S$ at 96 hours after reserpine; at that time levels of 5-HT in animals treated with reserpine only were repleted to at least 50% of reserpine-placebo rats in all these fractions. The significant changes in subcellular 5-HT after LSD ranged from 28 to 40% above reserpine treated animals (fig. 5).

**Effect of LSD on 5-HT in synaptosomal subfractions in reserpine-treated animals.** The 5-HT content of three synaptosomal subfractions was analyzed after an i.p. injection of 520 μg of LSD per kg b.wt.: Wp, $P_2V$ and Se. Measures in these fractions were made at 2, 4, 7 and 15 days after reserpine (fig. 6). In the Wp fraction, LSD had no significant effect on 5-HT until 7 days after reserpine, at a time when 5-HT levels were repleted to about 50% of placebo control; this increase occurred also at 15 days after reserpine.

In contrast, the crude vesicular fraction (which in the absence of reserpine had shown a striking and consistent 50% increase in 5-HT) showed no 5-HT increase after LSD at *any* of the times studied, although a 50% repletion was evident already at 4 days after reserpine. The significant LSD-induced 5-HT increases, in fact, were localized in the Se. This increase was statistically significant even at 2 days after reserpine as well as at 4, 7 and 15 days. On any of these days the 5-HT increase ranged from 21 to 47% above the values of reserpine-treated animals and each of the increases measured was significant ($P < .05$).

## Discussion

Although the precise mode of action of LSD still eludes us, the present work demonstrates clearly that the LSD-induced "enhanced binding" of 5-HT (Rosecrans *et al.*, 1967) is localized largely to a subcellular fraction rich in synaptic vesicles. This finding is additionally corroborated by the observation that pretreatment with reserpine abolished the 5-HT effect of LSD in the vesicular fraction for as long as 2 weeks after reserpine. Rather, with reserpine pretreatment the LSD-induced increase in 5-HT is localized to an intrasynaptosomally derived supernatant, revealing an unanticipated site for "enhanced binding" of 5-HT. Thus, in the unpretreated animal, we can conceive of the vesicle as the site retaining the LSD-induced increase in 5-HT, whereas a "juxtavesicular" fraction (Se) of the nerve-ending cytoplasm shows the drug-induced increase in 5-HT when vesicular storage is impaired by reserpine.

A significant increase in whole brain 5-HT had been found in reserpine-pretreated animals after LSD; this increase was clearly localized in the high-speed particulate of brain homogenate prepared from rats not treated with reserpine (Freedman, 1961a,b; Schanberg and Giarman, 1962) but also with LSD given at 24 hours postreserpine. In preliminary studies of whole brain 5-HT, we found a marked variability in the 5-HT response to reserpine alone as well as after LSD after pretreatment with reserpine. In an earlier report, we had distinguished a rat population of partial responders (about 20%) on the basis of percent loss of body weight (Halaris and Freedman, 1975); these rats showed significant 5-HT elevation after LSD at 24 hours postreserpine. However, if special attention was paid to preselecting fully responding animals, LSD did not significantly raise 5-HT in rat whole brain over its expected 60-minute period of action during the initial 24 hours after reserpine. The trend toward a 5-HT increase after LSD was apparent at 48 hours, and at 72 hours the increase was statistically significant. Obviously, a mixed population of rats had been used in the earlier studies.

Brain 5-HIAA, by contrast, was reduced by LSD as early as 12 hours after reserpine, when levels were increased 2-fold due to the action of reserpine. The LSD-induced 5-HIAA reduction was observed at all times after reserpine independent of an associated LSD-induced 5-HT increase. In the well documented absence of MAO inhibition by LSD (Freedman, 1961a, b; Collins *et al.*, 1970), the decrease in 5-HIAA had been attributed to enhanced particulate binding of 5-HT. With the finding that the 5-HT increase after LSD is mainly localized to the vesicular fraction, we would have expected at the time of reserpine-induced storage impairment that the portion of 5-HT associated with the enhanced binding would be rapidly metabolized by MAO and appear as an increase in 5-

HIAA. But rather than an increase in 5-HIAA, we found a decrease with LSD even at 12 hours postreserpine. This decrease suggests that a synaptosomal soluble macromolecule or element—normally present within the nerve-ending and other than the synaptic vesicle—might be accountable for the early inaccessibility of 5-HT to MAO as well as the juxtavesicular binding of 5-HT observed at 48 hours after reserpine and later.

In subcellular compartments, reserpine-induced 5-HT depletion was comparable in all crude fractions. Maximal 5-HT depletion (80–85%) occurred in all crude fractions (nuclear, mitochondrial, microsomal and true supernatant) 12 hours after reserpine, but levels must have been lower at earlier time points as indicated by whole brain data (Halaris and Freedman, 1975). Repletion rates, in contrast, varied among the crude fractions in that the true supernatant showed the fastest rate. In accord with previous data (Giarman *et al.*, 1964), half-maximal repletion occurred in the true supernatant at 48 hours after reserpine. The crude nuclear and microsomal fractions were slower; half-maximal repletion was evident around 72 hours after reserpine. The slowest rate among the crude fractions occurred in the crude mitochondria in which half-maximal repletion had not been reached even at 96 hours after reserpine. After LSD, significant 5-HT increases were not observed in crude subcellular fractions until 96 hours after reserpine.

The three synaptosomal subfractions did not differ significantly in repletion rates. However, the repletion rate of the end supernatant was considerably slower compared with the fast rate of repletion of the true supernatant which had also been studied by Giarman *et al.* (1964). The true supernatant consists mainly of soluble cell cytoplasm but excludes the nerve-endings which are "pinched-off" during homogenization. Thus, crude particulate fractions, which are slower to replete, contain the intrasynaptosomally derived end supernatant described here as well as the synaptic vesicles. Since the true supernatant repletes faster than the particulate fractions and since both particulates and supernatants can show a differential 5-HT elevation after LSD, we would have to conceive of sites other than the synaptic vesicles that are variably capable of 5-HT binding after LSD. For example, the free 5-HT may bind to proteins or other soluble macromolecules, which are normally present, in proportion to the amount of extravesicular amine. The proximity of the end supernatant to the vesicles, the depleting action of reserpine thereon and the ability to retain 5-HT after LSD ascribe a crucial role to the end supernatant. Perhaps the slow rate of repletion of the end supernatant reflects some "carrier" function—as if it could "yield" 5-HT to the maximally depleted and faster repleting stores. However, caution should be exercised since at present we cannot rule out the possibility that the end supernatant being "trapped" inside the nerve-ending may not be as readily accessible to the MAO inhibitor used in our *in vitro* preparation, as are the true supernatant or, for that matter, other subcellular particles. Studies of 5-HT turnover are crucial in determining differential synthesis and repletion rates in the two supernatant fractions.

In the Wp, a significant 5-HT increase did not occur until 7 days after reserpine again at a time when a 50% repletion was manifest. We attribute the 5-HT content and the LSD effect in this fraction to the presence of small undisrupted and also partially disrupted nerve-endings as revealed by electron microscopy. This fraction presumably contains "trapped" end supernatant. Interestingly, the Wp 5-HT is significantly increased by LSD only after reserpine pretreatment as is true for the end supernatant. The percent increase in both fractions is comparable, but the 5-HT increase in the "juxtavesicular" fraction is 5-fold greater. The lack of response to LSD in both fractions in the unpretreated animal must be due to the availability of intact vesicles which, in fact, showed the increase in 5-HT. Vesicular 5-HT did not increase after LSD even up to 15 days after pretreatment with reserpine although levels were 50% repleted by 4 days. The significant LSD-induced 5-HT increases were, in fact, localized to the end supernatant as early as 2 days after reserpine although 5-HT repletion had not reached 50% of control. Accordingly, the end supernatant seems to have some special capacity to bind or retain 5-HT even after reserpine.

It is not clear whether the presence of reserpine unmasks a 5-HT binding site normally present in the end supernatant. The question of a "soluble binding site" for 5-HT within nerve-endings has frequently been raised both theoretically and by data. That a number of

intraneuronal sites may display the capacity to bind 5-HT by a mechanism other than the $Mg^{++}$-ATP-dependent storage vesicle is suggested by a series of findings. Dahlström and Fuxe (1964), for example, demonstrated that 5-HT fluorescence in cell bodies and axons was enhanced after MAO inhibitors but electron microscopic examination failed to reveal the presence of subcellular particles to account for the increased fluorescence (Lenn, 1965; Fuxe *et al.*, 1966). Segawa (1970) reported 5-HT accumulation in vesicular fractions of rabbit brain after reserpine, a MAO inhibitor and 5-hydroxytryptophan. Recently Tissari (1975) and Tissari and Raunu (1975), noting that after MAO inhibition the infant rat brain accumulates 5-HT in relative absence of synaptic vesicles, concluded that brain 5-HT neurons may bind the amine by a mechanism other than the reserpine-sensitive storage granule. Unfortunately, the use of a MAO inhibitor in the above cited studies does not allow a direct comparison with the present findings.

Our results of an enhanced 5-HT binding after LSD in the end supernatant implicate a nonparticulate binding or storage form for 5-HT. Furthermore, they indicate that whatever component mechanism of LSD is responsible for the increase in 5-HT, the binding is semi-independent of the availability of intact storage granules. Binding of 5-HT to soluble, high affinity binding protein from nerve-endings of serotonergic tracts and its ion dependency has been documented (Tamir and Huang, 1974; Tamir *et al.*, 1976). Whether the LSD-induced "enhanced soluble binding" of 5-HT in the end supernatant reported here is linked to that protein remains to be seen. In view of the observed pharmacological responses of the juxtavesicular compartment, it is tempting to speculate whether it might be associated with the presynaptic serotonin receptor postulated by Haigler and Aghajanian (1974). This study of drug interactions and subsynaptosomal 5-HT does suggest a "juxtavesicular compartment" that may be of functional importance in the presynaptic regulation of 5-HT transport to enzymes, vesicles and membranes.

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