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THE EFFECT OF LSD-25 ON AMINES, AMINO-ACIDS, AND PROTEINS IN THE SNAIL (*HELIX POMATIA*) BRAIN

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ABSTRACT

1. The effect of LSD-25 on the content of amines, amino-acids, and proteins in the central ganglia of the snail *Helix pomatia* has been studied.
2. In very low concentrations the drug completely depleted an unidentified substance (possibly hydroxyproline), which was only present at certain times of the year.
3. LSD-25 treatment also resulted in a slight elevation of serotonin and tryptophan levels.
4. No effect was observed on the proteins and other amines and amino-acids.
5. The significance of these results is discussed.

BECAUSE of its psychotomimetic properties, and the fact that the drug is the most powerful hallucinogenic compound known to man, considerable interest and speculation have centered around the possible mode of action of LSD-25 (lysergic acid diethylamide) in the central nervous system. Gaddum's discovery in 1953 of an antagonistic physiological relationship between LSD-25 and serotonin (5-hydroxytryptamine) first attracted attention to the interaction of these two substances in the brain (*see* Smythies, 1970; Aghajanian, 1972). One of the best known actions of LSD-25 (Freedman, 1961; Giarman and Freedman, 1965) is the variation in the brain serotonin content which ensues after administration of this drug.

However, since LSD-25 is known to affect the behaviour patterns of a number of animals, e.g., nest-building behaviour in mice (Schneider and Chenoweth, 1970), open-field performance in rats (Dandiya, Gupta, Gupta, and Patni, 1969), T-maze test in mice (Stasik and Kidwell, 1969; Schechter and Rosecrans, 1972), it would seem probable that a number of other substances in the brain, in addition to serotonin, are influenced by the drug. Conclusive evidence has already been presented that LSD-25 affects the brain catecholamine levels (Smythies, 1970). Reports have also been published showing the drug to influence both the composition of proteins (Missere, Tonini, and Babbini, 1964; Piha, Bergström, Bergström, Uusitalo, and Oja, 1963) and steroids (Smythies, 1970).

In the invertebrates, LSD-25 has been demonstrated to mimic the excitatory action of serotonin on molluscan (e.g., *Helix pomatia*) heart (Welsh and McLoy, 1957), and muscle (Twarog, 1959) preparations. Cottrell (1970) analysed the effect of LSD-25 on a serotonergic synapse in the snail brain and observed that at a concentration of 100 μg. per ml. the drug blocked the excitatory action of serotonin, whilst it potentiated transmission at a concentration of 1 μg. per ml. This finding, in addition to the facts that LSD-25 causes different behavioural effects in the snail from reserpine (Mirolli and Welsh, 1964) and that the drug may influence axonal conduction in a special way (*see* Cottrell and Laverack, 1968), suggests that other biochemical substances in the snail nervous system may also be altered.

It was subsequently decided to study the effect of LSD-25 on the content of a number of substances in the snail (*Helix pomatia*) brain, noting that some of the best evidence suggesting serotonin to be a transmitter substance comes from studies on snail nervous tissue (Cottrell and Laverack, 1968; Gerschenfeld and Stefani, 1968; Cottrell, 1970, 1971;

Cottrell and Osborne, 1970; Osborne, 1972; Osborne and Cottrell, 1972). A sensitive microbiochemical procedure involving the use of dansyl chloride (*see* Neuhoff, 1971; Osborne, Briel, and Neuhoff, 1970) was used to analyse the amine and amino-acid composition, whilst the total protein of the brain was extracted in different ways (Grossfeld and Shooter, 1971; Waehneldt, Osborne, and Neuhoff, 1972) and examined by disc electrophoresis. Preliminary reports of this work have appeared elsewhere (Osborne, Maier, and Neuhoff, 1972; Osborne and Neuhoff, 1972).

MATERIALS AND METHODS

Helix pomatia were collected locally, kept in a glass tank at room temperature, and fed on lettuce.

In initial experiments the anterior aorta of the snail was canulated just before it enters the central ganglia and perfused either with physiological saline (Meng, 1960) containing LSD-25 (1–100 µg. per ml.) or with saline alone (control). The perfusion fluid was placed in a 1-ml. syringe, which was fixed to a micrometer holder, and the pressure adjusted so that 1 ml. fluid was perfused over 3 hours.

In most experiments circumoesophageal ganglia were dissected from a number of snails, allowed to equilibrate for 10 minutes in physiological snail saline, and then placed individually for 30 minutes in either 1 ml. saline (control) or saline containing varying amounts of LSD-25 (1–100 µg. per ml.). In some experiments circumoesophageal ganglion masses were preincubated for 1 hour in snail saline containing [^{14}C]glucose (Radiochemical, Amersham, 335 mc. per mmole, 0·2 mc. per ml. of saline) before exposing the nervous tissue to saline containing LSD-25.

Amines and Amino-acids

Briefly the procedure involves the reaction of aliphatic -NH_2 and -OH groups from extracted amines, amino-acids, and related compounds with dansyl chloride at alkaline *p*H, and the subsequent separation of dansyl derivatives on 3 × 3-cm. polyamide layers (*see* Osborne, Briel, and Neuhoff, 1970; Neuhoff, 1971; Osborne and Neuhoff, 1972). A single substance which contains two reactive groups (e.g., serotonin) can thus provide three possible dansyl compounds, namely N-, O-, and bis-forms. In practice the number of compounds formed depends on the experimental conditions (Briel, Neuhoff, and Maier, 1972), and in the instance of serotonin only two derivatives are formed (Neuhoff and Weise, 1970).

The suboesophageal ganglia of each circumoesophageal ganglionic mass were rapidly dissected, rinsed in physiological saline, blotted dry, and weighed. One–3 mg. of tissue from each subeosophageal ganglion mass were subsequently homogenized in 20–60 µl. of 0·05 *M* sodium bicarbonate, *p*H 10, and centrifuged for 15 minutes at about 20,000 *g*. The proteins in the supernatants were precipitated by the addition of equal volumes of cold acetone (20–60 µl.) and the precipitation completed by placing the solutions in a freezer (−20° C.) for 30 minutes. Thereafter each of the solutions was allowed to thaw, centrifuged once more for 15 minutes, and the supernatants then transferred to clean tubes. Equal volumes of solution and 5 µmoles/ml. of [^{14}C]dansyl chloride (Schwarz/Mann, Orangeburg, New York; specific activity 49 mc. per mmole) were mixed together and incubated in the dark for 30 minutes at 37° C. In experiments where circumoesophageal ganglia were initially preincubated in [^{14}C]glucose, the extracted amines and amino-acids were reacted with unlabelled dansyl chloride (1 mg. per ml. in acetone).

Aliquots of dansylated derivatives were then carefully applied to a corner of a 3 × 3 polyamide layer (Carl Schleicher & Schüll F 1700 micropolyamide) and developed in an ascending way. Water/formic acid (100 : 3, v/v) was the solvent used in the first dimension, and benzene/acetic acid (9 : 1, v/v) in the second dimension. Standard amounts of substances were added to extracts to assist in their identification. Autoradiograms of chromatograms were prepared using Agfa-Gevaert Rugzijde-Dos (exposure time 3 days).

Proteins

Circumoesophageal ganglia from 5–7 animals were homogenized in hypotonic buffer (0·01 *M* Tris-HCl, *p*H 7·2) to produce an approximately 5 per cent (w/v) suspension, and centrifuged at 100,000 *g* for 60 minutes. The hypotonic supernatant was then carefully removed with a syringe, and the remaining aqueous-soluble proteins in the pellet were extracted twice more with the same buffer. Thereafter the pellet was homogenized with 0·5 per cent Triton X-100, to release the Triton-soluble proteins, which were separated by centrifugation. Finally the residue was homogenized in 0·2 per cent SDS (sodium dodecyl sulphate), centrifuged once more at 100,000 *g* for 60 minutes, and the SDS-soluble proteins were carefully removed. The minute residual pellets, which consisted mainly of connective tissue and contained about 4 per cent of the total proteins (Waehneldt and others, 1972), were discarded.

Protein contents were determined according to the method described by Lowry, Rosebrough, Farr, and Randall (1951) with bovin serum albumin as a standard.

Graded gels were cast according to the procedure described by Grossfeld and Shooter (1971) for the electrophoresis of the hypotonic extract. They consisted of 4 per cent (0·1 ml.) acrylamide on top, 6 per cent (0·2 ml.) and 9 per cent (0·5 ml.)

in the middle, and 12 per cent (0·5 ml.) at the bottom. The gel tubes had an inner diameter of 5 mm. The upper buffer consisted of Tris (6·32 g.) and glycine (3·94 g.), made up to 1000 ml. with water, and the lower buffer of Tris (12·1 g.) and 1 *M* HCl (50 ml.), also made up to 1000 ml.

The Triton extract (after 12 hours of dialysis against 0·1 per cent SDS, *p*H 7·2, at room temperature) and SDS extracts were electrophoresed on 12 per cent polyacrylamide gels according to the method described by Waehneldt and Mandel (1972). As in the procedure used to fractionate the hypotonic extract, a concentrating gel was not employed.

The gels were generally loaded with 100–150 µg. proteins, and the fractionated proteins detected by staining the gels with Amido Black (0·3 per cent Amido Black in 10 per cent acetic acid). For analysis of glycoproteins the gels were loaded with more proteins (250–400 µg.) and stained with Periodic acid Schiff (PAS) reagent as described by Zacharias, Zell, Morrison, and Woodlock (1969).

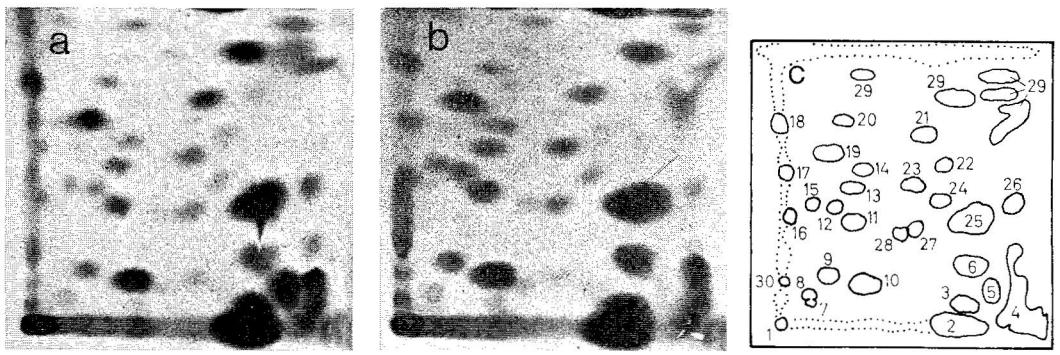

FIG. 1.—Autoradiograms of substances in (a) a control circumoesophageal ganglia extract and (b) an LSD-25-treated circumoesophageal extract, after being reacted with [^{14}C]dansyl chloride and chromatographed in two dimensions. The original single microchromatogram measured 3 × 3 cm., and was developed in the first dimension (horizontal direction) with water/formic acid (100 : 3 v/v) and in the second dimension (vertical direction) with benzene/acetic acid (9 : 1 v/v). The numbers on the map (c) correspond to the dansyl compounds which occur in each extract, as shown in *Table I.* Unmarked spots on chromatograms belong to impurities of the [^{14}C]dansyl chloride.

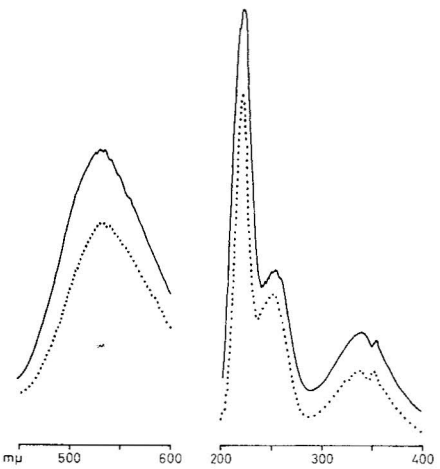

FIG. 2.—Emission spectrum (left) and excitation (right) of pure hydroxyproline (—) and unknown substance depleted by LSD-25 (...) in absolute ethanol. Both spectra were taken with 25 Å bandwidth on each of the two monochromators. For the emission spectrum the excitation was at 340 nm. and for the excitation spectrum the emission monochromator was at 530 nm.

RESULTS

AMINES AND AMINO-ACIDS

The substances which occur in the suboesophageal ganglia of the snail and which react with [^{14}C]dansyl chloride are shown in *Fig.* 1. A map of the [^{14}C]dansyl substances assists in the identification process. A single substance (U_I) which is present in nervous tissue of the controls (*Fig.* 1a) is seen to be completely absent from the LSD-25-treated animals (*Fig.* 1b). The precise nature of this substance, which occurs only in snails found after the winter hibernation, i.e., April and May, and which is completely reduced by as little as 1 µg. per ml. LSD-25, is not known, though the evidence available suggests that it may be hydroxyproline. The substance has the same chromatographic mobility as pure hydroxyproline, even when other solvent

systems are used, viz., ethyl acetate/methanol /acetic acid (20 : 1 : 1), benzene/methanol/ acetic acid (9 : 1 : 1), benzene/triethylamine (5 : 1), benzene/acetic acid (4 : 1), heptane/ *n*-butanol/acetic acid (3 : 3 : 1). In addition, examination of the excitation and emission spectra of dansyl hydroxyproline and the unknown substance (*see Fig.* 2) show them to be very similar.

To see whether LSD-25 specifically depleted the unknown substance, a number of other drugs were tested, namely *p*-chlorophenylalanine, reserpine, *α*-methyltyrosine, and bromolysergic diethylamide (BOL). It was found that each of these substances produced the same depleting effect at concentrations only greater than 100 μg. per ml.

Table I shows a comparison from ten different experiments of the percentage change of the radioactivity associated with each dansyl substance in the suboesophageal ganglia of *Helix pomatia* before and after LSD-25 treatment. An indication of the variation in results from one experiment to another is given in brackets as the average deviation from the mean. We do not consider changes of less than 5 per cent as significant; changes above this level most probably reflect real differences between the LSD-25-treated and control nervous tissue. Thus, changes in only two substances appear. Both the serotonin (15·2±2·8 per cent) and tryptophan (9·9± 2·3 per cent) levels are slightly elevated. Even when higher amounts of LSD-25 are

Table I.—Percentage Changes in Radioactivity associated with Dansyl Substances in *Helix* Suboesophageal Ganglia before and after LSD-25 Treatment

Spot No.	Substance	Percentage Change after LSD-25 Treatment (± for 10 different experiments)
1	Starting point	—
2	Dansyl-OH (not counted)	—
3	Glutamic and aspartic acids	4·2 (±2) increase
4	Glutamine, serine, threonine, argenine, ϵ-lysine, α-aminohistidine, *N*-cystine	4·8 (±3·5) increase
5	Unknown U_1	100 decrease
6	Glycine	2·9 (±2·6) decrease
7	*N*-Serotonin	15·2 (±2·8) increase
17	bis-Serotonin	
8	Tryptophan	9·9 (±2·3) increase
9	bis-Lysine	3·9 (±2) decrease
10	bis-Ornithine	4·9 (±2) decrease
11	Phenylalanine	2·7 (±1·8) increase
12	bis-Histidine	2·9 (±2·9) decrease
13	Leucine	3·3 (±1·5) increase
14	Isoleucine	2·9 (±1·3) increase
15	Unknown U_2	3·5 (±1·2) decrease
16	bis-Tyrosine	4·8 (±2·3) decrease
18	5-Hydroxyindole	4·8 (±2·6) decrease
19	Unknown U_3	3·9 (±1·8) increase
20	Unknown U_4	3·3 (±1·7) increase
21	Proline	2·1 (±1·9) decrease
22	Unknown U_5	2·9 (±1·7) decrease
23	Valine	2·1 (±1·8) decrease
24	GABA	3·9 (±1·4) increase
25	Alanine, dansyl-NH_2	4·9 (±2·5) decrease
26	Ethanolamine	4·4 (±2·6) decrease
27	Unknown U_6	3·3 (±2·1) increase
28	Methionine	4·5 (±3·9) increase
29	Excess dansyl chloride (not counted)	—
30	bis-5-Hydroxytryptophan	4·9 (±3·3) decrease

used (100 μg. per ml.), only these two substances are affected, and to a similar extent.

The effect of LSD-25 (1 μg. per ml.) on the radioactivity of [^{14}C]glucose incorporated into nervous tissue glutamine, glutamic acid, alanine, and aspartic acid is shown in *Fig.* 3. It can be seen that no alterations were recorded; nor did higher amounts of LSD-25 (100 μg. per ml.) have any effect.

Proteins

Fig. 4 gives the protein patterns of hypotonic extracts (*Fig.* 4a, a′), Triton extracts (*Fig.* 4b, b′), and SDS-extracts (*Fig.* 4c, c′) from LSD-25-treated and control nervous tissue after electrophoretic fractionation and staining with Amido Black. The regions marked by the bars to the right of the gels display strong glycoprotein bands when stained with PAS reagent.

Examination of the characteristic protein patterns from each extract in more than ten different experiments showed that LSD-25 had no obvious effect upon the proteins. Even when very high amounts of LSD-25 were used (1000 μg. per ml.), no alterations could be seen.

DISCUSSION

Our findings show that LSD-25 (in very low concentrations) specifically depletes a substance which occurs in nervous tissue of snails only in the period after the winter hibernation. This substance in its dansylated form has the same chromatographic mobility as hydroxyproline in a number of different solvent systems, in addition to having very similar excitation and emission spectra. However, the information derived from the spectra analysis is not sufficient for any conclusions to be made. We have also injected LSD-25 into 5-day-old rats which have hydroxyproline in their nervous system (Agrawal, Davis, and Himwich, 1966), but failed to register any effect upon the substance. It is difficult to explain why LSD-25 so dramatically depletes this single substance in the snail nervous tissue, yet has little influence upon the other substances which react with dansyl chloride. Furthermore the effect, although specific for LSD-25 in very low concentrations (1 μg. per ml.), can be brought about by higher amounts of other drugs, i.e., *p*-chlorophenylalanine, reserpine, *α*-methyl-*m*-tyrosine, and BOL.

The other effects of LSD-25 on the amine and amino-acid content of the snail nervous tissue, even in high concentrations (100 μg. per ml.), are to increase the serotonin and tryptophan levels slightly. It is known from studies on vertebrate nervous tissue that the drug elevates the serotonin (Freedman, 1961; Freedman and Giarman, 1962; Schanberg and Giarman, 1962) and tryptophan (Tonge and Leonard, 1970) levels, but has no effect upon the 5-hydroxytryptophan (Freedman and Giarman, 1962) content. It thus appears as if the snail nervous tissue is influenced in precisely the same way. Although these results would suggest that LSD-25 alters the contents of two substances which are involved in the metabolism of serotonin, it is possible that only one substance is specifically affected and that the influenced substance is involved in a feed-back mechanism on to the second substance. There is appreciable evidence that brain tryptophan levels directly influence

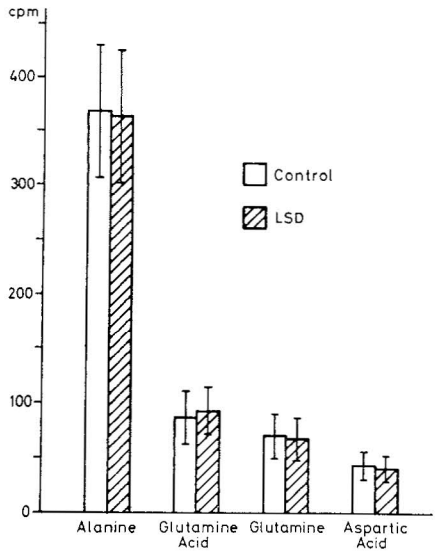

Fig. 3.—Effect of LSD-25 on the radioactivity incorporated into certain amino-acids from [^{14}C]glucose.

the turnover time of serotonin in certain situations (Bloom and Costa, 1971). Unlike vertebrate nervous tissue where LSD-25 elevates the tyrosine level (Tonge and Leonard, 1970), we could find no significant change in the tyrosine level of the snail nervous system.

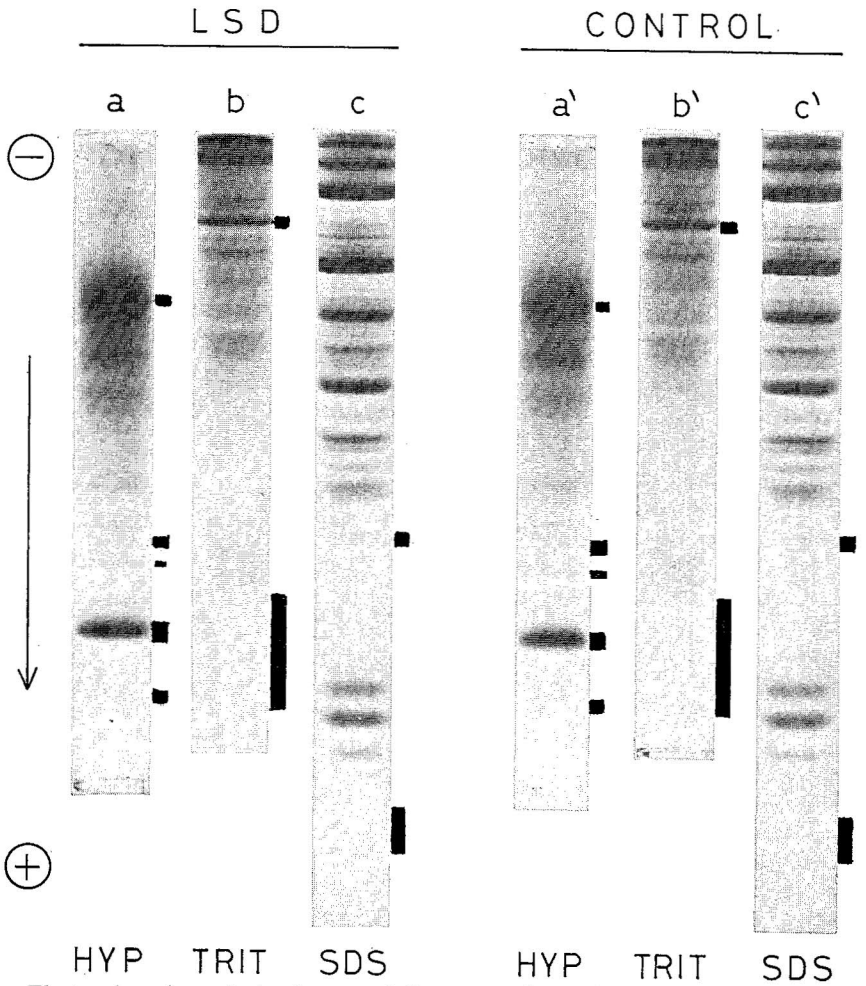

FIG. 4.—Electrophoretic analysis of sequentially extracted protein pools (HYP = hypotonic, TRIT = Triton X-100, SDS = sodium dodecyl sulphate) of LSD-25-treated (LSD) and untreated (CONTROL) animals. The hypotonic extracts were separated on graded gels (cf. text); Triton X-100 extracts and SDS extracts were electrophoresed on 12 per cent acrylamide gels in the presence of SDS. Lengths of gel columns were 74, 69, and 85 mm. for hypotonic, Triton X-100, and SDS extracts respectively. Bars to the right of each gel indicate PAS-positive material.

In vertebrate nervous tissue LSD-25 is known to alter the levels of the catecholamine content (Smythies, 1970). We, however, did not study the effect of the drug upon these substances in the snail brain because of the difficulty in distinguishing the different dansyl derivatives of the various catecholamines on the microchromatograms (*see*, e.g., Neuhoff, 1971). However, experiments were designed where radioactivity was incorporated into glutamic acid, aspartic acid, alanine, and glutamine (preincubating the tissue in radioactive glucose) so that the specific effect of LSD-25 on these possible transmitter substances could be tested. The data clearly demonstrate that LSD-25 has no effect. In

similarly designed experiments it has been shown that lithium ions primarily caused release of radioactive glutamic acid, followed by glutamine and aspartic acid, whilst the content of radioactive alanine remained constant (Osborne, Maier, and Neuhoff, 1972).

Though LSD-25 has only a small or negative effect on enzyme reactions (Greig and Gibbons, 1959), reports have shown that in vertebrate tissue the drug changes not only the protein electrophoretic pattern (Missere, Tonini, and Babbini, 1964) but also the rate of incorporation of amino-acids into brain proteins (Piha and others, 1963). In the present study it is demonstrated by the electrophoretic analysis of sequentially extractable proteins (hypotonic, Triton, and SDS) that in the snail LSD-25 does not influence the normal protein distribution. Even when comparatively high amounts of the drug were used (100 µg. per ml.) no alterations were recorded. It should however be pointed out that the methods used are probably not sufficiently sensitive to detect minute quantitative changes in the individual proteins.

In this report we have attempted to study the possible site of action of LSD-25 by analysing the effect of the drug upon the contents of amino-acids, amines (excluding the catecholamines), and proteins of the snail nervous system. In general it would appear that LSD-25 does not influence the proteins or amino-acids, though there is a slight increase in the tryptophan level. In contrast a single substance, as yet unidentified and occurring in the nervous system only at certain times of the year, is completely reduced. The only other substance affected is serotonin, which is elevated to a greater extent than tryptophan.

ACKNOWLEDGEMENTS

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