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Histochemical Studies on the Mechanism of Action of the Hallucinogens d-Lysergic Acid Diethylamide Tartrate (LSD-25) and d-2-Bromo-Lysergic Acid Tartrate (BOL-148) in Rat Brain

By

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With 18 Figures in the Text

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Introduction

The use of lysergic acid diethylamide and d-2-bromo-lysergic acid tartrate (BOL-148) as psycho-mimetic drugs in the study of schizophrenia has been well-known for a long time. The clinical reports by different investigators indicate that in man LSD-25 causes temporary psychic disturbances characterized by different types of hallucinations, disturbances of thought, speech, behaviour and perception, together with some autonomic disturbances, such as, rise of blood pressure, heart rate, body temperature, lacrimation and dilation of pupils. BOL-148 causes similar mental changes though to a milder degree (ISBELL et al. 1959; BARON et al. 1958; HOFFER et al. 1959). Although a number of workers have studied these drugs to determine their sites of action in the central nervous system, little is understood about their mode of action, or degree of inhibition of different enzymes in different parts of the brain. A review of the literature shows a wide variation in the techniques used and results obtained, especially with regard to the effects of such drugs on pseudo-cholinesterase (THOMSON et al. 1954; FOLDES et al. 1959; POLONI and MAFAZZONI 1952; GOLDBERGER 1961).

Although the inhibitory action of LSD-25 on the hydrolysis of acetyl thiocholine (ACthCh) in the brain has been explained by previous workers, its effects on the activity of non-specific cholinesterase have not been adequately dealt with. It may be pointed out here that although it has been shown that BOL-148 produced symptoms clinically allied to those of LSD-25 but to a much lesser degree (BARON et al. 1958; HOFFER et al. 1959; ISBELL 1959), its effects on acetylcholinesterase and non-specific cholinesterase, especially with histochemical methods, have not been studied adequately.

PEARSE (1960) indicated that an important function of amine oxidase in the animal body is probably the detoxication of poisonous amines produced in the intestine by bacterial decarboxylation of amino acids, and it may also play a major part in the detoxication of adrenalin at adrenergic nerve endings and other sites. The concept of cholinergic and non-cholinergic nerve endings is well known and it was thought important to study the localization of monoamine oxidase at these sites and to compare the results with those obtained after treatment with LSD-25 and BOL-148.

Although the effects of LSD on adrenalin and adrenochrome metabolism have been observed biochemically by Costa and Zetler (1959), Hoffer and Callbeck (1960), they have not been studied by histochemical methods.

Material and methods

In the present investigation, both the normal sites of localization of specific cholinesterase, non-specific cholinesterase and monoamine oxidase in the rat brain and the effects of drugs such as d-lysergic acid diethylamide tartrate (LSD-25) and d-2-bromo-lysergic acid tartrate (BOL-148) on these enzymes have been studied by various methods.

Young adult albino rats were killed by decapitation or by bleeding through cut carotid and jugular veins, and the brains were immediately removed by careful dissection. Fresh frozen sections, 10 μ thick, were cut in the cryostat and mounted on pre-cleaned coverslips. The experiments were carried out in three series with careful controls to standardize the results and to avoid any alterations due to variations in the technique.

1. Both LSD-25 and BOL-148 were administered by either intraperitoneal, intravenous or intracardiac injection in different animals, in the dose of 0.1 mg/kg of body weight, and the animals were killed within a half to one hour after injection, time of sacrifice being decided by observation of their change in behaviour. The brains of normal rats of the same age and untreated with drugs, were studied as controls.

2. Sections from an untreated normal rat brain were placed in Coplin jars in incubating media both with and without the drug, at 37° C, the jar without drugs being the control in this case. Drugs were used in the amount of 0.1 mg/10 ml of the final incubating mixture.

3. Some sections were first placed in normal saline containing the drug (0.1 mg/10 ml of saline for both LSD-25 and BOL-148) and then finally placed in the incubating mixture, while others were incubated for the same period of time without previous treatment by either drug. The latter sections were taken as controls.

The enzyme localization was studied by the methods of Coupland and Holmes (1957), and Gerebtzoff's modification of Koelle's method (1959) for both specific and non-specific cholinesterases, and that of Glenner et al. (1957), for monoamine oxidase. The incubation times for each enzyme localization were determined as 40 min for specific cholinesterase and 7 to 8 hours for non-specific cholinesterase. A longer period of incubation showed needle crystals and diffusion of enzymes into surrounding tissues. In the case of monoamine oxidase the optimum period of incubation was found to be from one to two hours.

In the case of specific cholinesterase controls were prepared by means of specific inhibition by treating the sections with eserine sulphate before incubating in the final incubating mixture. The control tests for other enzymes were performed by incubating the tissues without the substrate in the incubation mixtures.

Some of the sections were counterstained with toluidin blue as well as Nuclear Fast red dyes for better identification of the cells. In all cases, the experimentals and controls were treated identically as regards the periods of incubation, thickness of sections used and preparation of the incubating medium.

Results

Our attention was directed particularly to the cerebral cortex and some of the basal nuclei (caudate and thalamic). In the controls the specific cholinesterase reaction showed a positive result after 40 min incubation, longer periods resulted in the formation of needle-like crystals, especially in the basal ganglia which were extremely rich in specific cholinesterase. In the cerebral cortex, the plexiform layer showed the most intense reaction (Figs. 3 and 4), next in order being the pyramidal cells (Fig. 5) and then the granular layers (both inner and outer). The laminae multiformis showed the least reaction. The positive reaction was localized in the synapses and cell processes and positive areas were also noted on the surfaces of the pyramidal cells (Fig. 5). We were unable to locate any positive reaction in the cytoplasm of the granule and pyramidal cells.

It was interesting to note that the neurons of the caudate and thalamic nuclei showed an intracytoplasmic reaction for specific cholinesterase (Fig. 9).

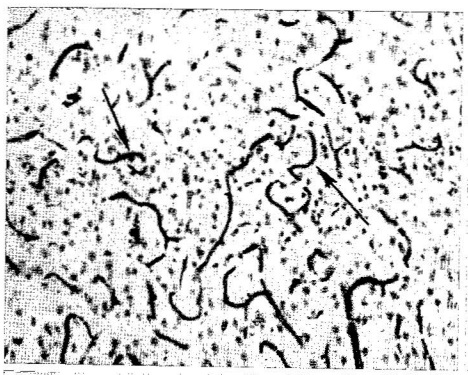


Fig.1

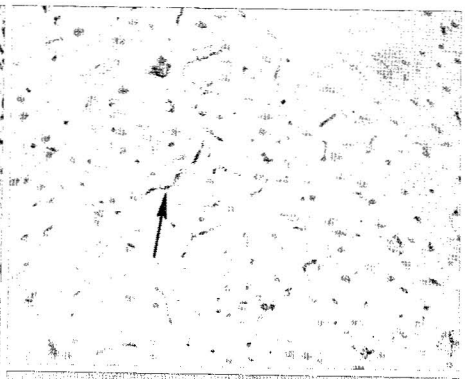


Fig.2

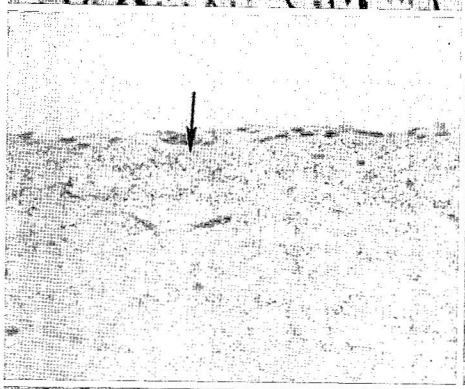


Fig.3

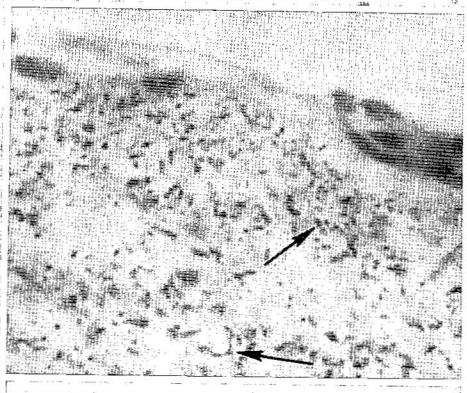


Fig.4

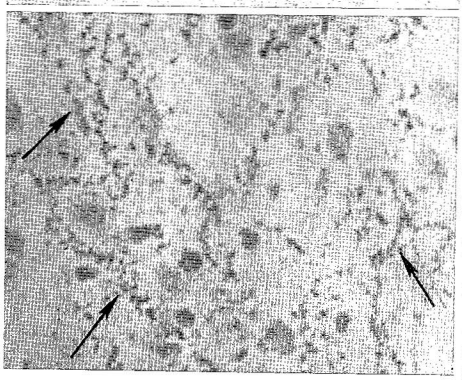


Fig.5

Fig.6

Fig. 1. Cerebral cortex of the rat, fresh frozen section. Gerebtzoff's non-specific cholinesterase technique using butyryl thiocholine iodide as substrate, connterstained with Nuclear Fast red. Note the intense positive reaction in the blood vessels (arrows) whereas the rest of the cortex is completely negative. × 136

Fig. 2. Cerebral cortex of the rat. Non-specific cholinesterase reaction, counterstained with Nuclear Fast red. Fresh frozen sections were treated with BOL-148 in normal saline before incubation. Note the intensity of the reaction in the blood vessels is very much reduced (arrow). Compare with Fig. 1. ×136

Fig. 3. Plexiform, outer-granular and outer-pyramidal cell layers of rat cerebral cortex. Specific cholinesterase reaction using acetyl thiocholine iodide as substrate, counterstained with Nuclear Fast red. Note intense positive reaction in the plexiform layer (arrow), whereas the intensity is very much less in layers II and III. ×340

Fig. 4. Higher magnification of the Fig. 3. Shows small round and oval shaped positive areas of true cholinesterase reaction (arrows) in the plexiform layer, probably representing nerve terminals. ×1360

Fig. 5. Layer V of the cerebral cortex. Specific cholinesterase reaction counterstained with Nuclear Fast red. Note the positive areas of reaction on the surface of the pyramidal cells and their process (arrows). Cytoplasm is negative. ×510

Fig. 6. Plexiform layer of cerebral cortex of the rat. Specific cholinesterase reaction. Fresh frozen sections were pretreated with LSD-25 in normal saline before incubation. Note inhibition of the true cholinesterase activity (arrow). Compare with the normal reaction in Fig. 3 and 4 of plexiform layer. ×340

Non-specific cholinesterase was exclusively localized in the blood vessels (Fig. 1) and in the white matter of the brain. The neurons were completely negative.

Fig.7

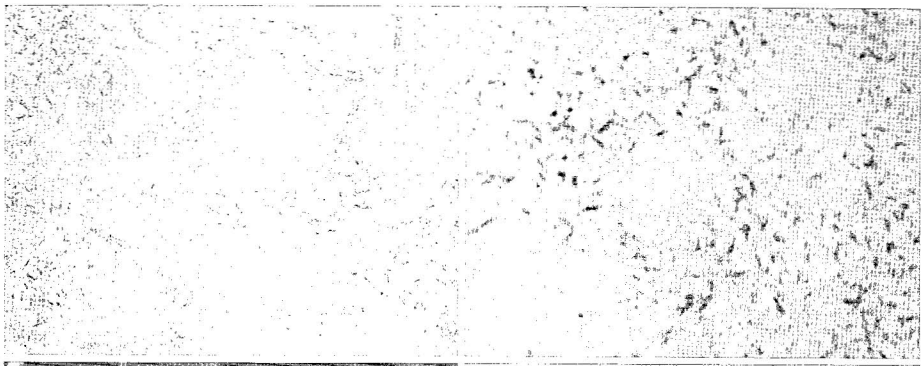


Fig.8

Fig.9

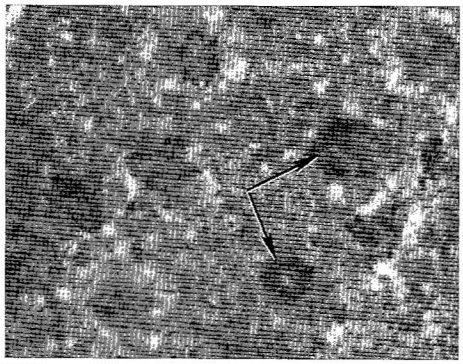


Fig.10

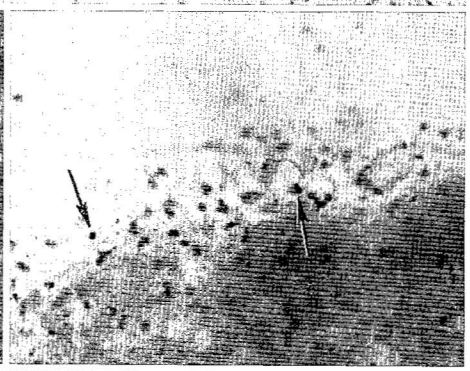


Fig.11

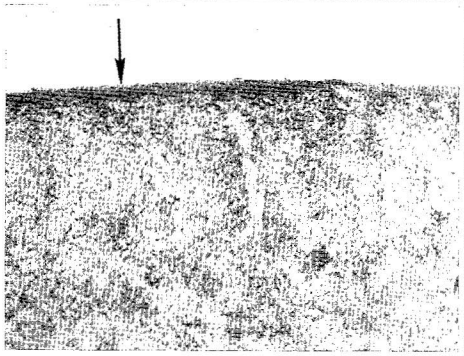


Fig.12

Fig. 7. Plexiform, outer-granular and outer pyramidal cell layers of rat cerebral cortex. Specific cholinesterase reaction in animals, following administration of LSD-25. Note great inhibition of enzyme activity in these layers of specific cholinesterase. Compare with normal reaction (Fig. 3, 4 and 5). ×340

Fig. 8. Layer V of rat cerebral cortex. Specific cholinesterase reaction in animals following administration of BOL-148. Note great inhibition of reaction in pyramidal cells. Compare with normal reaction (Fig. 5). ×1360

Fig. 9. Caudate nucleus of the rat. Specific cholinesterase reaction. Note intense positive reaction in the caudate nucleus. Neurons of the caudate nucleus show intracytoplasmic reaction (arrows). ×1360

Fig. 10. Plexiform layer of the rat cerebral cortex at a higher magnification. Monoamine oxidase reaction. Note the small oval shaped, positive areas of reaction (arrows) probably representing the nerve terminals in this layer (Compare Fig. 4 and 15 which also show such positive areas), suggesting their similarity. ×1360

Fig. 11. Cerebral cortex of rat. Monoamine oxidase reaction. Note intense positive reaction in the plexiform layer (arrow). ×136

Fig. 12. Layer V of cerebral cortex. Monoamine oxidase reaction. Note the positive reaction in the nerve fibers and neurons (arrow). Note that the nerve fibers, show a knotted type of reaction over their whole lengths, probably indicating the synaptic contacts on the nerve fibres as well as the dendritic spines. ×340

The reaction for monoamine oxidase was mainly localized in the form of strongly positive areas on the peripheries of cells in the synaptic regions, and in the cells processes the reaction

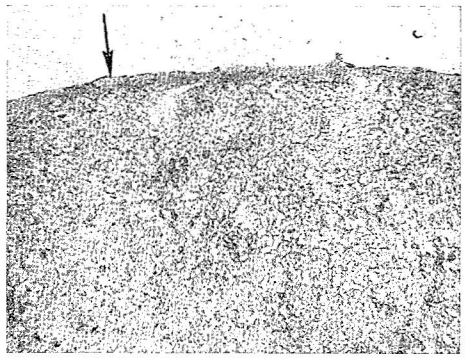


Fig.13

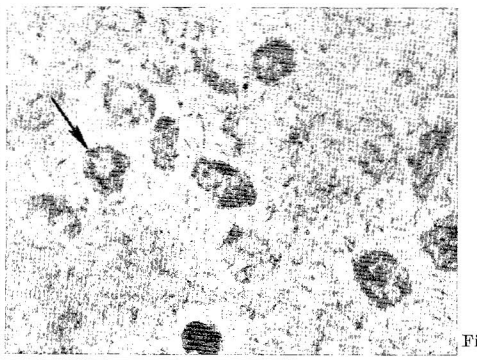


Fig.14

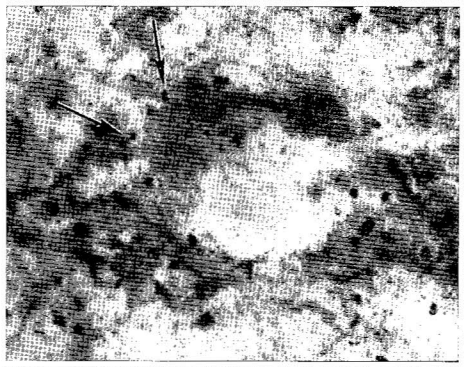


Fig.15

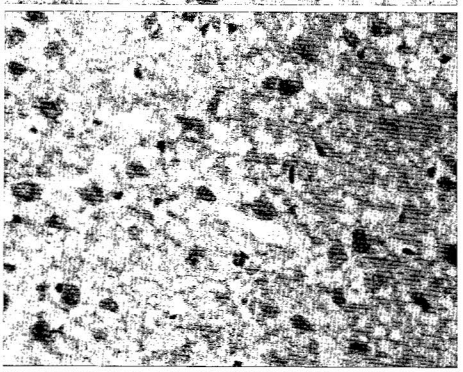


Fig.16

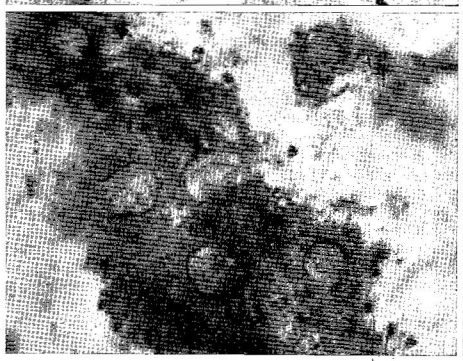


Fig.17

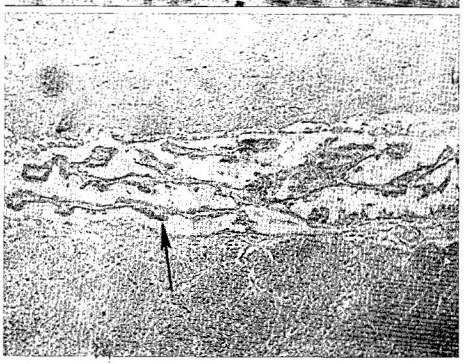


Fig.18

Fig. 13. Plexiform layer of the rat cerebral cortex. Monoamine oxidase reaction. Fresh frozen sections pretreated with BOL-148 in normal saline. Note complete inhibition of monoamine oxidase reaction in plexiform layer (arrow) as well as in other layers of the cortex. ×1360

Fig. 14. Caudate nucleus of the rat. Specific cholinesterase reaction. Fresh frozen sections pretreated with LSD-25 in normal saline. Note the inhibition of enzyme activity. Compare with normal activity at same timings of incubation (Fig. 9). ×1360

Fig. 15. Pyramidal cells of layer V of rat cerebral cortex, monoamine oxidase reaction. Note diffuse positive reaction in cytoplasm and small, rounded or oval areas of reaction on the surface (arrow) probably indicating nerve endings on the surface of the cell membrane. Compare these identical areas in the plexiform layer (Figs. 4 and 10). ×1360

Fig. 16. Layer V of rat cerebral cortex. Monoamine oxidase reaction. Fresh frozen sections pretreated with BOL-148 in normal saline, counterstained with Nuclear Fast red after incubation. Note the complete absence of reaction in neuron as well as in their process. Compare with normal reaction (Fig. 12). ×340

Fig. 17. Choroid plexus from rat's lateral ventricle. Monoamine oxidase reaction. Note the intense diffuse type of reaction in the cytoplasm of the choroid plexus cells. ×1360

Fig. 18. Choroid plexus from rat's lateral ventricle. Monoamine oxidase reaction. Fresh frozen sections pretreated with LSD-25. Note the almost complete absence of reaction in the choroid plexus cells (arrow). ×136

was most intense at the synaptic junctions (Fig. 15). The processes of different cells could be traced from their cells of origin to their arborization either on the cell surface or with processes of another cell (Figs. 10, 12 and 15). The plexiform layer of the cerebral cortex was strongly positive including the spherules of the collateral branches of Cajal cells (Figs. 10 and 11). The reaction in the pyramidal and granule cells was characterized by a positive reaction on the cell membranes in the regions of the synapses (Fig. 15) with light, diffuse and non-granular reactions in the cytoplasm. The cells of the caudate nucleus and the thalamus showed more intense reactions than the cortical neurons and the periphery of the cytoplasm in these cells also showed a positive reaction. The choroid plexus of the lateral ventricle showed a strong positive which was granular and diffuse (Fig. 17).

In the tissues treated with LSD-25 a general inhibition of reaction of specific cholinesterase was observed (Fig. 6 and 7). The reaction in the plexiform layer was reduced and a lesser degree of reaction was consistently noticed in the synapses, cell processes and on cell surfaces of pyramidal cells. The inhibition was much more profound in the neurons of the caudate and thalamic nuclei (Fig. 14), the mild positive areas of reaction being limited to the periphery of the cytoplasm. In the case of non-specifiic cholinesterase a reduction in the intensity of reaction was noted in both the capillaries and white matter.

The inhibition of the reactions of both acetyl cholinesterase and non-specific cholinesterase was also observed with BOL-148 (Fig. 2 and 8). In spite of careful observation complete inhibition was never noted in any of the experiments either for specific or non-specific cholinesterase.

The effects of LSD-25 and BOL-148 on monoamine oxidase reaction were much more profound than was expected. It was observed that there was an almost complete inhibition of the reaction on the cell membrane, synaptic junctions and cell processes of the pyramidal and granule cells (Figs. 16). The plexiform layer also exhibited a profound inhibition (Fig. 13). Both the normal reaction and the inhibitory effects of the drugs were more marked in the basal nuclei than elsewhere. The choroid plexus also showed a profound inhibition of this enzyme in LSD-25 and BOL-148 treated tissues (Fig. 18).

In the three series of experiments, better and more uniform results were obtained in the third series, in which the tissues were pre-treated with the drugs in normal saline before putting them into the incubating solutions.

Discussion

In the present study the effects of LSD-25 and BOL-148 on the normal reactions of specific and non-specific cholinesterases and monoamine oxidase in the cerebral cortex and basal nuclei of rat were studied histochemically in three series of experiments: 1. By administration of the drugs to the animals direct (intraperitoneally, intracardially or intravenously). 2. By incubating the section in the substrate mixed with the drug. 3. By pre-treating the sections with the drug in normal saline solution, before incubation in the appropriate substrate.

In our preparations the most regular and consistent results were obtained with the third series of experiments. It appears that the enzymes were sufficiently inactivated by the drugs before the tissues were placed in the substrates.

The results of experiments in the first series, though constant, showed less inhibition, which may be due to a smaller concentration of the drugs in the blood, as well as to simultaneous and continuous reformation of the enzymes; this possibility is completely eliminated in the third series of experiments. In the second series, results were less uniform. The degree of inhibition was much less for all three enzymes, compared to the other two series of experiments. Most probably this was due to the simultaneous action of the substrate and the drugs upon the enzyme systems, and probably also to the substrates acting faster than the drugs during the period of incubation.

In all cases our observations indicated that specific cholinesterase was localized mainly at synaptic regions and the cell processes. There was no detectable intracytoplasmic localization of this enzyme in pyramidal or granule cells of the cerebral cortex, although we constantly observed a positive reaction in the cytoplasm of the neurons of the basal nuclei, especially in the caudate nucleus. Our observations are similar to those of KOELLE and STEINER (1956) and GEREBTZOFF (1959), and it appears to us that the intracytoplasmic localization in pyramidal cells reported by GOLDBERGER (1961) may be due to the diffusion of enzymes caused by longer incubation periods.

Non-specifiic cholinesterase was invariably present in the blood vessels and in the white matter of the brain, this is in agreement with the results of KOELLE and STEINER (1956) and GEREBTZOFF (1959), whereas GOLDBERGER (1961) reported a positive reaction in the blood vessels only.

THOMSON et al. (1954) in a series of biochemical studies found that the degree of inhibition by LSD-25 of specific cholinesterase was only $10^0/_0$ whereas that of non-specific cholinesterase was $50^0/_0$ in human subjects. In our present observations inhibition of specific as well as non-specific cholinesterases was observed to be of equal degree, at least as far as could be determined by subjective appraisal of histochemical preparations.

Monoamine oxidase reaction was observed mostly in synaptic regions on the cell surfaces and cells processes, although the cytoplasm also showed a mild positive reaction. The reaction was characteristically localized in the boutons terminaux, described by MEYER and MEYER (1945) in cerebral cortex. The neurons of the basal nuclei, including the thalamus, exhibited a stronger reaction than the cortical cells. Although a marked inhibition by LSD-25 and BOL-148 was noted in the cerebral cortex, a more profound inhibition was observed in the cells of the caudate nucleus and thalamus.

It has been reported by DE ROBERTIS et al. (1962), that the cerebral cortex not only contains cholinergic endings but also non-cholinergic ones. In our present study we constantly observed the localization of monoamine oxidase, an enzyme partially responsible for the destruction of adrenalin, in the synaptic regions. Both LSD-25 and BOL-148 profoundly inhibited this monoamine oxidase reaction in the synapses, as it also did in the case of cholinesterases.

According to COSTA and ZETLER (1959) LSD-25, even in high doses (100 mg/kg and 125 mg/kg), potentiated the effect of adrenalin, whereas according to HOFFER et al. (1959) plasma adrenochrome levels and conversion of adrenalin to adrenochrome were increased by LSD-25. The latter workers also observed that the ability of the blood to destroy adrenochrome was reduced by LSD-25 and they thought LSD-25 inhibited an enzyme responsible for conversion of adrenochrome into dihydroxyindoles.

GREEN and RICHTER (1937) have described adrenalin as inducing a vigorous oxygen uptake when added in low concentrations to the reconstructed lactic and malic dehydrogenase system of heart muscle. This effect was found to be due to a red coloured oxidation product: adrenochrome; which acts as a respiration carrier. FRIEDHEIM (1933) found that the respiration of rabbit red blood corpuscles increased by $50^0/_0$ by addition of the red compound obtained by oxidizing adrenalin (the oxidation product being adrenochrome); so it is highly probable that the

raise in adrenochrome level with LSD-25 (HOFFER et al. 1959) may increase the respiration in brain tissues which may result in hyperactivity of the neurons producing hallucinations.

It has also been observed (NANDY et al. 1963) that both LSD-25 and BOL-148 inhibit the reaction of monoamine oxidase in hepatic cells and it has been suggested that the drugs raise the adrenochrome level of the blood by depressing the body mechanism destroying adrenalin. Thus excess of adrenalin accumulates in the body which is probably oxidized to adrenochrome producing the hallucinogenic effect described above.

It may be suggested here that the non-cholinergic synapses are possibly adrenergic in nature wherever monoamine oxidase is located; in a similar way to the location of acetyl cholinesterase at cholinergic nerve endings. The normal positive reaction of the basal nuclei and thalamus was stronger than that of the cortical area and the degree of inhibition was also much greater than in the cerebral cortex. It is possible that the richness of specific cholinesterase and monoamine oxidase in these nuclei is due to their important function in regulating and transmitting impulses from the sub-cortical level to the cerebral cortex and the inhibitory action of LSD-25 and BOL-148 on these centers may set up a release phenomenon which enables a greater stream of impulses to reach the cortex and disturb the finer balance of cortical functions, producing various psychic symptoms.

It is well-known that acetylcholine facilitates the synaptic transmission of impulses and that acetylcholinesterase hydrolyses the acetylcholine and thereby controls the transmission of excessive impulses. The inhibitory action of LSD-25 and BOL-148 on specific cholinesterase is probably due to the interference of the hydrolysis of acetylcholine by acetyl cholinesterase. The inhibitory effect of LSD-25 and BOL-148 on the hydrolysis of acetylcholine in various areas of human and rabbit brain was investigated by FOLDES et al. (1959). Those workers are of the opinion that LSD-25 and BOL-148 completely inhibit the hydrolysis of acetylcholine in a concentration of $3^{-3} \times 10^{-5}$ M in most areas of human and rabbit brain.

ELKES (1961) observed by electrophysiological methods that one effect of LSD-25 was to sensitize the animal inordinately to its environment, and suggested that it may be due to the lowering of the threshold for external stimuli.

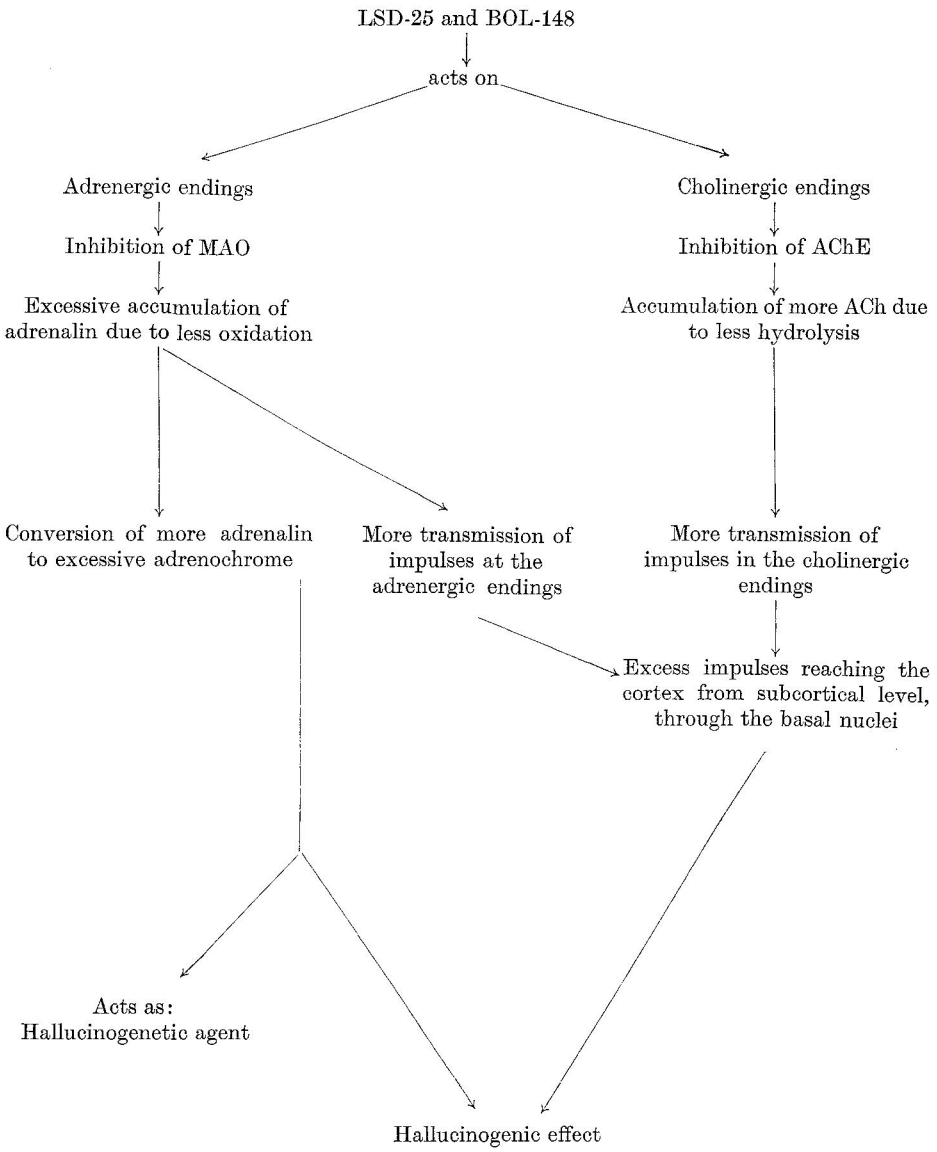
As suggested by our experiments the probable mechanism of action of LSD-25 and BOL-148 acting as hallucinogens may be summarized as follows:

(Schematic see p. 37.)

Summary

The normal localization of specific and non-specific cholinesterases was studied in rat brains by Gerebtzoff's modification of Koelle's method (1959) and by the COUPLAND and HOLMES method (1957); and monoamine oxidase by the technique of GLENNER et al. (1957).

The effects of LSD-25 and BOL-148 have been studied on the above enzymes and it has been observed that all these reactions are inhibited by both drugs. It has been pointed out that the degree of positive reaction for acetyl cholinesterase and monoamine oxidase is more intense in the basal ganglia and thalamus and that the inhibitory effect of the drugs was also profound in these regions. It has



been suggested that these drugs inactivate the cholinesterases and monoamine oxidase, thus preventing acetylcholine and adrenalin destruction which controls synaptic transmission of impulses.

The profound inhibition of acetyl cholinesterase in the thalamus and basal nuclei may set up a phenomenon allowing excessive impulses to enter the cerebral cortical centers from the subcortical level, producing the state of psychosis found by the experimental administration of the drug. It has also been suggested that the inhibition of monoamine oxidase results in more accumulation of adrenalin which is probably oxidized to adrenochrome, the latter acting as an hallucinogen in the body.

Zusammenfassung

Die normale Lokalisation der spezifischen und unspezifischen Cholinesterasen im Rattengehirn wurde mit Gerebtzoffs Modifikation der Koelleschen Methode (1959) sowie mit der Coupland- und der Holmes-Methode (1957) untersucht; die Monoamino-Oxydase mit der Technik von Glenner et al. (1957).

Die Wirkungen von LSD-25 und BOL-148 auf die genannten Enzyme werden studiert. Es ergibt sich, daß alle Reaktionen durch beide Drogen gehemmt werden. Es wird dargelegt, daß der Grad der positiven Reaktion auf Acetylcholinesterase und Monoamino-Oxidase in den Stammhirnkernen und im Thalamus am höchsten und auch die hemmende Wirkung der Drogen in denselben Gebieten eine beträchtliche ist. Es wird vermutet, daß diese Pharmaka die Cholinesterase und die Monoamino-Oxidase inaktivieren und auf diese Weise den Abbau von Acetylcholin und Adrenalin, welches die synaptische Impulsübertragung beeinflußt, verhindern.

Die weitgehende Hemmung der Acetylcholinesterase im Thalamus und in den Stammhirnkernen dürfte ein Ausschüttungsphänomen hervorrufen, welches eine Impulsüberflutung der Großhirnrindenzentren vom subcorticalen Niveau ermöglicht, wodurch der bei der experimentellen Anwendung dieser Drogen zu beobachtende psychotische Zustand hervorgerufen wird. Weiter wird vermutet, daß die Hemmung der Monoamino-Oxydase eine Anhäufung von Adrenalin zur Folge hat, das wahrscheinlich zu Adrenochrom oxydiert wird, welches seinerseits im Körper als Halluzinogen wirksam wird.

Acknowledgement

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