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THE EFFECT OF LSD ON THE HISTOLOGY AND ULTRASTRUCTURE OF THE NEUROEPITHELIUM OF YOUNG CHICK EMBRYOS: A STEREOLOGICAL STUDY

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SUMMARY

Five control chick embryos and fifteen exposed to LSD at concentrations of 2.5, 12.5 and 50 μg/ml were examined. The morphometric analysis performed at the light microscope level shows that (*1*) the section area of the neural tube increases with the two highest doses of lysergic acid diethylamide (LSD) employed, (*2*) the volume fraction of the intercellular spaces decreases with the two highest doses of LSD, (*3*) the volume fraction of the nuclei in the neural tube is not modified with any concentrations of LSD employed, (*4*) the volume fraction of the cytoplasm in the neural tube increases with all three concentrations, and (*5*) the nucleo-cytoplasmic ratio decreases with all three doses employed. Moreover, at the ultrastructural level, it was found that (*1*) the volume fraction of mitochondria in the cytoplasm decreases at doses of 12.5 and 50 μg/ml (*2*) the surface to volume ratio of the mitochondria is unchanged with any of the concentrations of LSD employed and (*3*) the surface density of the endoplasmic reticulum in the cytoplasm increases only with a dose of 2.5 μg/ml of LSD.

INTRODUCTION

The effects of LSD have been investigated in a wide variety of biological models. These include plant material [1,2], insects [3,4] and several rodents [5,8]. However, the mechanism of action of the drug is still subject to much controversy; notably those effects of the drug that touch upon

Abbreviation: LSD, lysergic acid diethylamide.

carcinogenesis, mutagenicity or teratogenicity [9]. In 1971, chick embryos incubated in vitro were used by Hart and Greene [10] as a system for testing the effect of LSD. These authors showed that 50 and 100 μg doses of LSD disturbed the fusion of neural folds in the neurulating embryo and that the 50 μg dose reduced the mean number of somites in the treated specimens. Using much lower concentrations (0.5 and 2.5 μg/ml) one of us [11] later confirmed that LSD retarded the segmentation of mesoderm into somites in the chick embryo growing in vitro. Finally, Hart [12] proposed that, between doses of 25 and 50 μg, there exists a dose related effect of the drug on the reduction in the number of somites formed under LSD treatment.

We have now turned to the analysis of the effects of various doses of LSD on several histological and ultrastructural parameters. We report here the results obtained in an extensive stereological study of the effects of LSD on the neuroepithelium of young chick embryos.

METHODS

This work is based on the study of four groups of specimens: three groups of embryos exposed to LSD at concentrations of 2.5, 12.5 and 50 μg/ml and a group of control embryos incubated in vitro. All embryos were of the chicken *Gallus domesticus*. They were developed in ovo until the 5-somite stage (stage 8^+ according to Hamburger and Hamilton [13]), whence they were explanted and incubated at 38°C for 5 h on Spratt's medium [14] to which LSD was added. Control embryos, also explanted at stage 8^+, were manipulated in the same way and incubated in vitro on similar media without LSD. The embryos were allowed to reach the stage 10^-.

Specimens were fixed for 60 min in a 1.25% phosphate buffered glutaraldehyde and post-fixed for 60 min in a 1% solution of phosphate-buffered osmium tetroxide. Following dehydration in an ethanol series the specimens were embedded in epon. 1 μm thick sections, stained with a 1% aqueous solution of toluidine blue were used for light microscopy. Thin sections for electron microscopy were cut on an LKB Ultrotome I and stained with 1% uranyl acetate and lead citrate. The sections were examined with a Siemens Elmiskop 1A electron microscope.

As shown in Figs. 1 and 2, sections were cut rigorously (*1*) in the anterior part of the cephalic region; (*2*) behind the cephalic region; (*3*) in the first pair of somites region; (*4*) in the ninth pair of somites region; (*5*) through the node of Hensen.

In light microscopy five parameters were studied, these are: the section area of the neural tube; the volume fraction of the intercellular spaces in the neural tube; the volume fraction of the nuclei in the neural tube; the volume fraction of the cytoplasm in the neural tube; the nucleo-cytoplasmic ratio. To calculate the section area of the neural tube, sections were projected and delineated on constant density paper which was cut and weighted [15]. Moreover the point counting method of Glagolev [16] was used to determine (*1*) the nucleo-cytoplasmic ratio, (*2*) the volume fraction of the

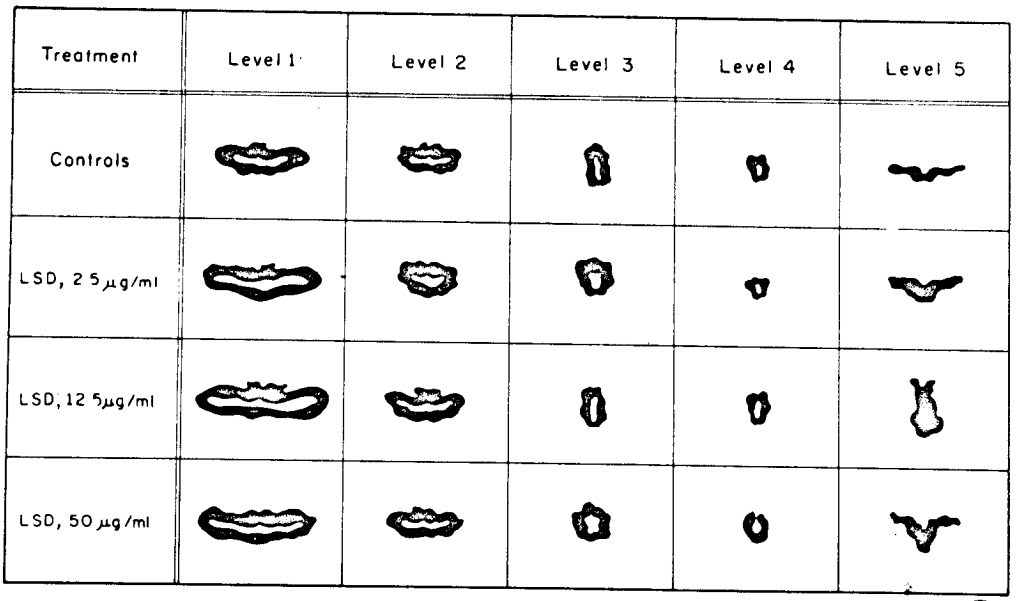

Fig. 1. General aspect of the cross sections (drawn to scale) cut at various levels along the length of the control embryos and of those exposed to LSD at concentrations of 2.5, 12.5 and 50 µg/ml.

intercellular spaces, that of the nuclei and of the cytoplasm in the neural tube. In making these measurements, the test system used was a square line lattice in which each intersection between the lines was considered as a point. Depending on the size of the section 4 to 25 micrographs were taken at magnification 1000 × in such a way as to cover the whole surface of the neural tube. On each micrograph superimposed with the test system we counted the number of points falling on the nuclei, the cytoplasm and the intercellular spaces.

In electron microscopy three parameters were studied, these are: the volume fraction of the mitochondria in the cytoplasm; the surface to volume ratio of the mitochondria; the surface density of the endoplasmic reticulum. These ultrastructural parameters, measured on embryos sectioned at level 2 only, were determined according to Glagolev [16] for the volumetric density and according to Saltikov [17] for the surface density. For each embryo we chose three squares of the grid to be representative of the whole area of the neural tube section: (*1*) one square showing a supra-chordal field of the neural tube, (2) one square exhibiting a field of the neural crest, and (3) one square showing a region intermediate between 1 and 2. 20 to 25 equally spaced micrographs were taken randomly in each square examined at a magnification of 12 000 ×. On each micrograph superimposed with a square line lattice we counted (*1*) the number of points falling on the sections of the

mitochondria, (2) the number of points over the cytoplasm, (3) the number of intersections of the horizontal lines of the lattice with the external membrane of the mitochondria, and (4) the number of the intersections between the horizontal lines of the lattice and the edges of the granular endoplasmic reticulum. The theoretical [18] and practical [19,20] aspects of the stereological methods summarized here have already been described in detail.

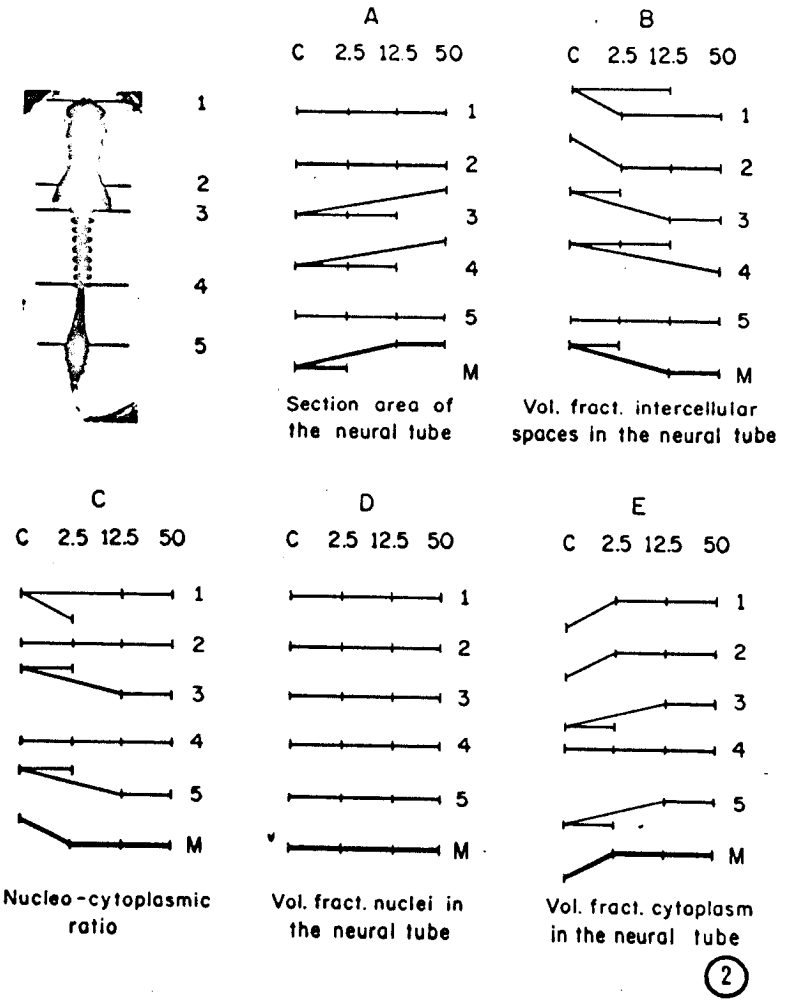

Fig. 2. These curves show the effect produced by LSD (doses 2.5, 12.5 and 50 μg/ml) at the different levels (1 to 5) for each of the parameters (A to E) studied by light microscopy. In each set of curves C represents the control embryos. The mean curve (see METHODS) is indicated by M.

In order to evaluate the effect of LSD along the cephalo-caudal axis, the values obtained at the different levels throughout the length of the embryos were compared with three non-parametric tests. These are Friedman's m ranking test [21], also Nemenyi's [22] and Page's [22]. The Dunnett test [23] was used to study the effects of the various doses of LSD at a given level. All comparisons were considered significant at a level of $p \leqslant 0.05$.

The curves plotted illustrate the differential effects of various doses of LSD at each individual level. Here, the comparisons between groups are based on Dunnett's multiple comparison test [23] for comparing several treatments with a control ($p \leqslant 0.05$). However, in order to further clarify and to give a more rapid picture of the results obtained, the data related to the different levels along the length of the embryos were coumpounded as follows: (*1*) in each group, we calculated the mean derived from the values measured at the five levels in every embryo; (*2*) the mean values found for the five embryos in each group were submitted to a Dunnett test [23] so as to determine the effect of the three concentrations of LSD employed. In other words, using data related to the individual level in each group of embryos, a value was derived which represents an average section cut transversely in an average embryo belonging to each group. These results, pertaining to the five parameters studied in light microscopy, are shown in the sixth curve, labelled M, in Fig. 2.

RESULTS

Section area of the neural tube

The sixth curve in Fig. 2A shows that the section area of the neural tube, in embryos treated with LSD at concentrations of 12.5 and 50 μg/ml, increases significantly as opposed to the control embryos. Yet the other curves illustrating this parameter indicate that only level 3 and 4 are significantly sensitive to LSD. It is observed further that only the highest concentration tested is effective in increasing the surface area of these levels.

Moreover, as shown in Fig. 3A the section area of the neural tube, throughout the cephalo-caudal axis of the embryos exposed to any of the concentrations of LSD, decreases as it does also in control embryos at the same developmental stage.

Volume fraction of the intercellular spaces in the neural tube

The mean curve in Fig. 2B shows that the volume fraction of the intercellular spaces decreases significantly with LSD doses of 12.5 and 50 μg/ml. Individual curves show that levels 1 and 2 are the most sensitive; indeed they respond even to a 2.5 μg/ml concentration. Photomicrographs taken at such levels in the control embryos (Fig. 5) and in those treated with a 2.5 μg/ml concentration (Fig. 6) clearly suggest a reduction in the intercellular spaces under LSD exposure. Level 3 is affected by the two higher concentrations, level 4 by only the highest dose and level 5 is not influenced significantly by any of the concentrations of LSD employed. However, perhaps one infelicity

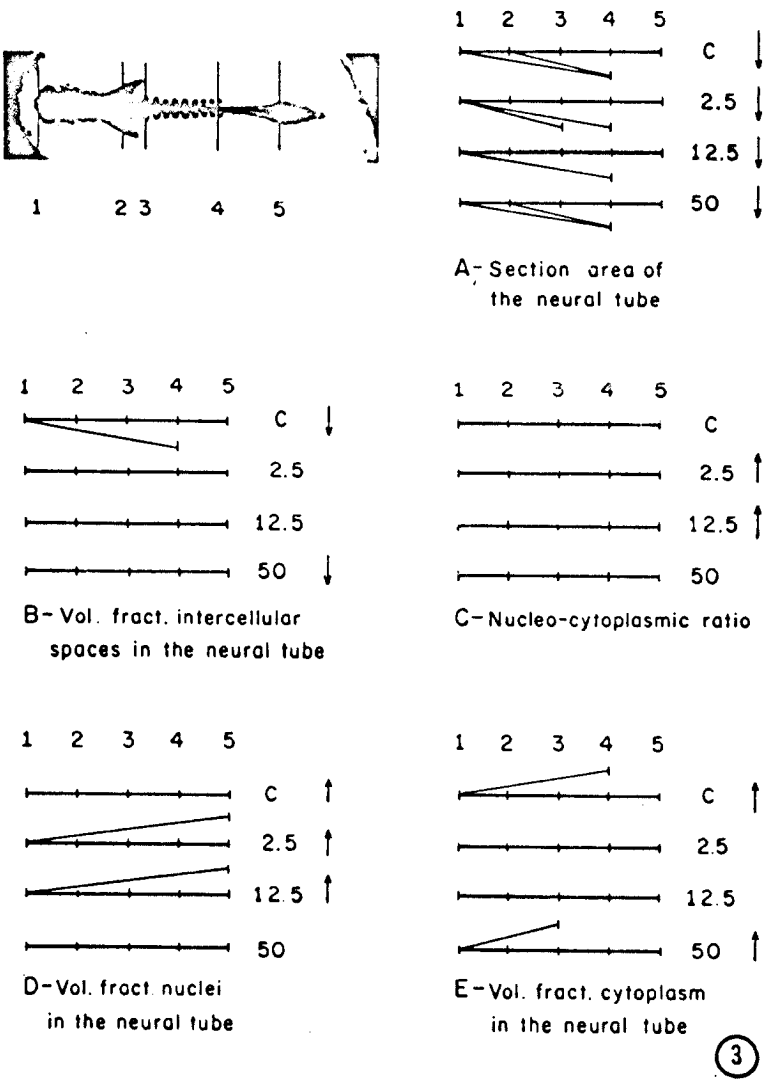

Fig. 3. These curves show the effects which the different concentrations of LSD employed produce on the cephalo-caudal differentiation of each parameter (A to E) studied in light microscopy. Levels (1 to 5) are indicated and, in each set of curves C represents the control embryos. The arrows indicate the increasing (upwards) or decreasing (downwards) tendency of the values.

is apparent here in that at level 1 a dose of 2.5 μg/ml appears to be effective in decreasing the volume fraction of the intercellular spaces while the higher dose of 12.5 μg/ml is not.

Finally, irrespective of the concentration of LSD employed, the volume

TABLE I

MEAN VALUES ± STANDARD ERROR OBTAINED AT EACH LEVEL IN BOTH CONTROL AND LSD-TREATED EMBRYOS

The underlined figures indicate the mean values ± standard error derived from the mean values measured at the five levels in each embryo.

Parameter	Level	Controls	2.5 μg/ml LSD	12.5 μg/ml LSD	50 μg/ml LSD
Section area of the neural tube	1	0.0407 ± 0.0056	0.0456 ± 0.0044	0.0607 ± 0.0063	0.0523 ± 0.0045
	2	0.0266 ± 0.0016	0.0218 ± 0.0017	0.0303 ± 0.0015	0.0318 ± 0.0043
	3	0.0155 ± 0.0008	0.0140 ± 0.0023	0.0193 ± 0.0010	0.0243 ± 0.0026
	4	0.0086 ± 0.0006	0.0096 ± 0.0008	0.0096 ± 0.0007	0.0114 ± 0.0007
	5	0.0157 ± 0.0030	0.0180 ± 0.0017	0.0190 ± 0.0022	0.0213 ± 0.0026
	Mean	0.0211 ± 0.0020	0.0218 ± 0.0018	0.0275 ± 0.0015	0.0282 ± 0.0013
Vol. fract. intercellular spaces in neural tube	1	0.15 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
	2	0.12 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	3	0.11 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
	4	0.10 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	0.05 ± 0.00
	5	0.10 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
	Mean	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.07 ± 0.00
Nucleocyto-plasmic ratio	1	0.34 ± 0.01	0.28 ± 0.01	0.30 ± 0.02	0.30 ± 0.01
	2	0.35 ± 0.01	0.32 ± 0.02	0.31 ± 0.00	0.31 ± 0.01
	3	0.36 ± 0.01	0.33 ± 0.02	0.28 ± 0.02	0.30 ± 0.02
	4	0.31 ± 0.02	0.34 ± 0.02	0.33 ± 0.01	0.30 ± 0.03
	5	0.38 ± 0.01	0.37 ± 0.02	0.32 ± 0.00	0.33 ± 0.01
	Mean	0.35 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.31 ± 0.00
Vol. fract. nuclei in neural tube	1	0.22 ± 0.01	0.20 ± 0.00	0.20 ± 0.01	0.21 ± 0.01
	2	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.00	0.22 ± 0.00
	3	0.24 ± 0.00	0.23 ± 0.01	0.21 ± 0.01	0.22 ± 0.01
	4	0.22 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.22 ± 0.01
	5	0.24 ± 0.00	0.25 ± 0.01	0.23 ± 0.00	0.23 ± 0.01
	Mean	0.23 ± 0.01	0.22 ± 0.00	0.21 ± 0.00	0.22 ± 0.00
Vol. fract. cytoplasm in neural tube	1	0.63 ± 0.01	0.71 ± 0.01	0.68 ± 0.01	0.69 ± 0.01
	2	0.65 ± 0.01	0.69 ± 0.01	0.72 ± 0.01	0.71 ± 0.01
	3	0.69 ± 0.01	0.70 ± 0.01	0.74 ± 0.02	0.73 ± 0.01
	4	0.72 ± 0.02	0.70 ± 0.01	0.74 ± 0.01	0.72 ± 0.02
	5	0.65 ± 0.01	0.68 ± 0.01	0.74 ± 0.02	0.72 ± 0.01
	Mean	0.66 ± 0.01	0.70 ± 0.01	0.71 ± 0.01	0.71 ± 0.01

fraction of the intercellular spaces remains unchanged along the cephalo-caudal axis of the treated embryos whereas it diminishes between level 1 and 4 in the control specimens (Fig. 3B). In fact (see Table I), it was found that in treated specimens, irrespective of the dose used, the volume fraction of the intracellular spaces was for each level always as small as that calculated at level 4 in the control specimens, with one exception. Indeed the value of this parameter was smaller in embryos exposed to 50 μg/ml of LSD.

Nucleo-cytoplasmic ratio

When considering the nucleo-cytoplasmic ratio (Fig. 2C), the mean curve indicates that all of the concentrations of LSD employed have reduced the value of this parameter. Here again, as individual curves show, level 1 is found to be sensitive to a dose of 2.5 μg/ml but not to the other concentrations. At no other level was the nucleo-cytoplasmic ratio significantly decreased by this low concentration of LSD.

Turning to the effect of LSD on the cephalo-caudal differentiation, it is observed that the nucleo-cytoplasmic ratio (Fig. 3C) is constant, from one level to the other, for all the concentrations of LSD employed just as it is constant throughout the length of the control embryos. Also, the values for the embryos exposed to 2.5 and 12.5 μg/ml of LSD show a tendency to increase as the sections are analysed from the cephalic to the caudal region of the embryos.

Volume fraction of the nuclei in the neural tube

Statistical analysis indicates that the volume fraction of nuclei, as measured throughout the length of the LSD-treated embryos (and at all concentrations), is not different from that which is found in the control embryos of the same age (Fig. 2D). However, the volume fraction of nuclei (Fig. 3D) increases from level 1 to level 5 in embryos exposed to 2.5 and 12.5 μg/ml of LSD, while it remains constant at the 50 μg/ml concentration. It should be noted that the values obtained for this parameter in the control embryos show a tendency to increase from the cephalic to the caudal region of the specimens.

Volume fraction of the cytoplasm in the neural tube

The mean curve in Fig. 2E shows that, when compared to control embryos, the volume fraction of the cytoplasm of LSD-treated specimens increases with any of the doses of LSD employed. Individual curves suggest that only level 4 is not influenced by these concentrations of LSD.

Moreover (Fig. 3E), the volume fraction of the cytoplasm in embryos exposed to the concentrations of 2.5 and 12.5 μg/ml remains constant from the cephalic to the caudal region, while it increases in embryos treated with LSD at the 50 μg/ml concentration. An increase in the values obtained for this same parameter in the control embryos was also detected between level 1 and 4.

TABLE II

MEAN VALUES ± STANDARD ERROR OBTAINED FOR THE ULTRASTRUCTURAL PARAMETERS AT LEVEL 2 IN BOTH CONTROL AND LSD-TREATED EMBRYOS

Parameter	Controls	LSD; 2.5 μg/ml	LSD; 12.5 μg/ml	LSD; 50 μg/ml
Volume fraction of mitochondria in cytoplasm	0.082 ± 0.008	0.070 ± 0.002	0.060 ± 0.004	0.058 ± 0.003
Surface density of endoplasmic reticulum in cytoplasm	0.31 ± 0.02	0.46 ± 0.04	0.36 ± 0.03	0.36 ± 0.01
Surface to volume ratio of mitochondria	7.5 ± 0.3	8.1 ± 0.6	8.2 ± 0.4	8.4 ± 0.2

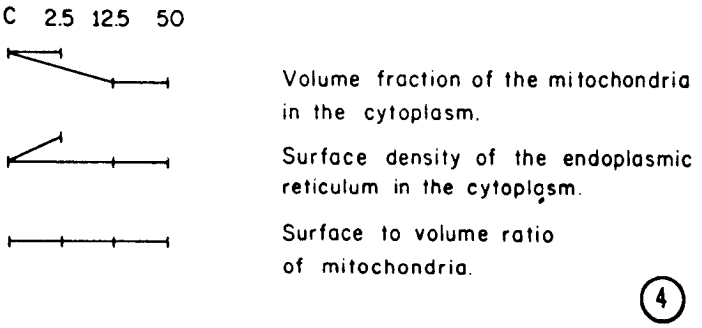

Fig. 4. These curves show the effect produced by each of the concentrations of LSD employed on the three parameters which were measured in electron microscopy. C stands for control embryos.

Ultrastructural parameters

The stereological study of the electron micrographs (Table II) showed that the volume fraction of mitochondria in the cytoplasm (Fig. 4) decreased when LSD was used at concentrations of 12.5 and 50 μg/ml. The comparison of electron-micrographs from control embryos (Fig. 7) with others taken from specimens treated with a 50 μg/ml concentration (Fig. 8) suggests, at first glance, that there is a decrease in the size of mitochondria under LSD exposure. However, the use of stereological methods revealed that the surface to volume ratio of mitochondria remained unchanged irrespective of the LSD concentration employed (Fig. 4), a clear indication that the size of the organelle has not been modified. Finally, the surface density of the endoplasmic reticulum in the cytoplasm increased at the 2.5 μg/ml concentration

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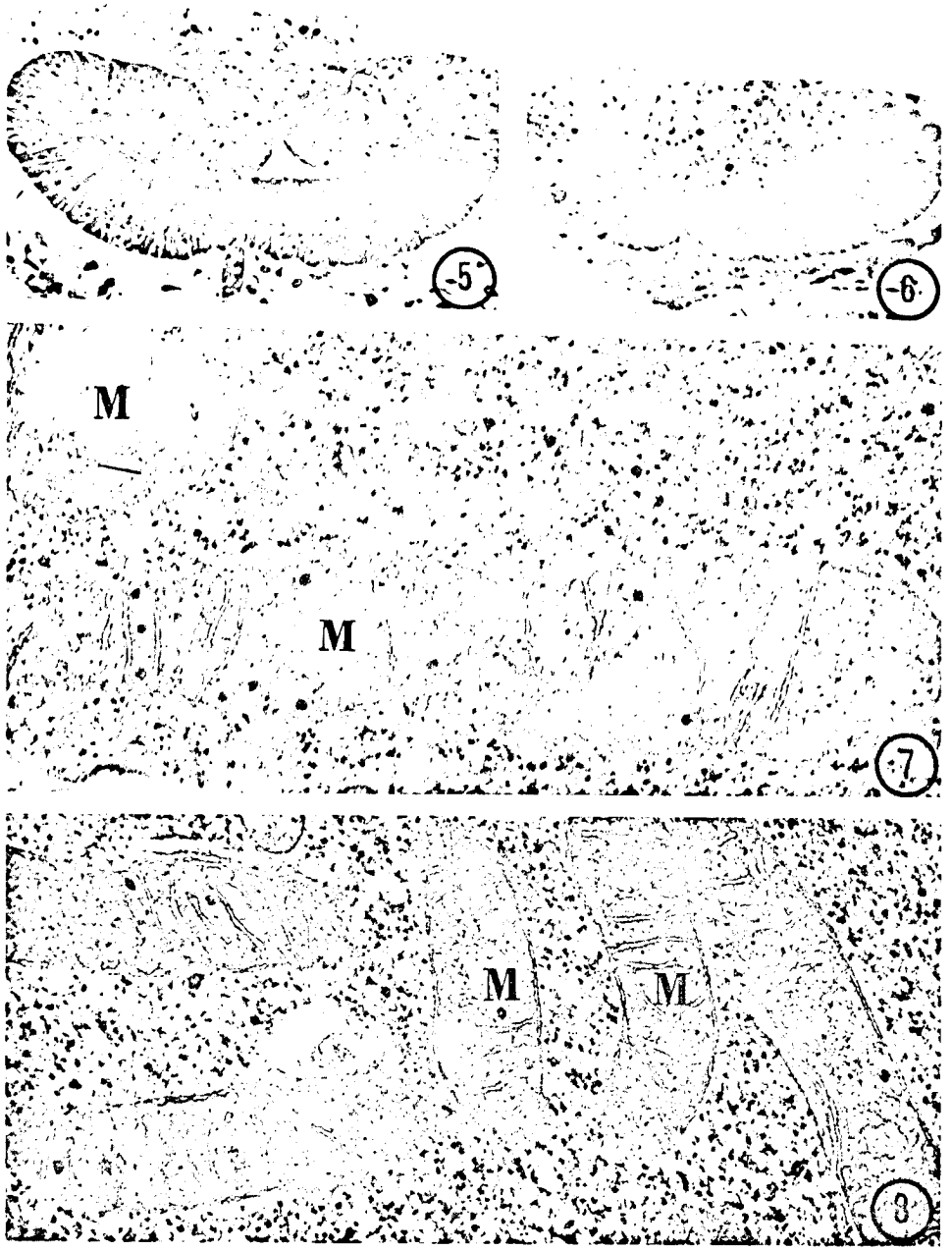
Fig. 5. Light micrograph of a section cut transversely behind the cephalic region (level 2) of a stage 10^- in vitro incubated embryo. ca. × 500.

Fig. 6. Light micrograph of a section cut transversely at level 2 in a chicken embryo exposed to a 2.5 μg/ml concentration of LSD. When compared with Fig. 5, this illustration suggests that there is a reduction in the amount of the intercellular spaces. ca. × 500.

Fig. 7. Medium power electron micrograph of part of two mitochondria (M) taken from a stage 10^- in vitro incubated embryo. × 42 000.

Fig. 8. This electron micrograph taken from a specimen exposed to a 50 μg/ml concentration of LSD suggests, at first glance, that the drug induces a decrease in the size of mitochondria (M). × 42 000.

(Fig. 4). For this same parameter the data obtained in embryos exposed to LSD at 12.5 and 50 μg/ml were not significantly different from the values measured in control embryos.



DISCUSSION

Our results indicate that LSD influences both histological and ultrastructural parameters in the neuroepithelium of young chick embryos. The stereological methods used are sensitive, accurate and they permit the detection of minute discrepancies. The data gathered in this morphometric study were submitted to statistical comparisons the results of which are illustrated in the form of simple curves. Occasionally these comparisons bring out inconsistencies; for example, the finding that a dose of 12.5 μg/ml of LSD does not significantly modify the volume fraction of intercellular spaces in the neural tube (Fig. 2B) while concentrations of 2.5 or 50 μg/ml both decrease the value of this parameter. In such incongruent cases the usefulness of a mean curve becomes evident. Any effect of LSD at a given level will be determinant only if it is sustained at other levels. It should be stressed that the mean curve cannot bring out any information which is not already implicit at one level; it can only pare down some discrepancies. Yet such a situation has not been encountered. Only one mean curve is totally flat (Fig. 2D), a clear reflection of the isolated values making up the individual curves which are all flat themselves. At worst, in our material, the mean curve can only mask the effects induced by the weakest concentration of LSD employed. This would seem to be the case with the volume fraction of the intercellular spaces in the neural tube (Fig. 2B).

Another inconsistency arises in relation to the nucleo-cytoplasmic ratio (Fig. 2C), which diminishes significantly with a dose of 2.5 μg/ml at level 1, but which is not affected at higher concentrations. Finally, a similar occurrence of a marked and isolated effect of the lowest dose of LSD is noted in relation to the surface density of the endoplasmic reticulum in the cytoplasm (Fig. 4C).

If we consider only the mean curves it is evident that, when compared to what is found in control embryos, concentrations of 12.5 and 50 μg/ml induce an increase in the section area of the neural tube (Fig. 2A). This increase could be explained if LSD (*1*) induced cell proliferation, (*2*) widens the intercellular spaces, (*3*) increases the volume fraction of the cytoplasm or if it produces any combination thereof. In order to test the first possibility, counts of nuclei (results not presented here) were undertaken; these showed that the number of cells is not increased by exposure to LSD. As for the volume fraction of the intercellular spaces we found that it decreases with both of the highest concentrations (Fig. 2B). In fact Fig. 3B shows that in control embryos the volume fraction of the intercellular spaces decreases more and more as sections are moving towards level 4. Such a reduction is no more apparent with exposure to LSD. This is because the intercellular spaces are as small at level 1 to 3 in treated embryos as they are at level 4 in the control specimens. Therefore changes in the volume of the intercellular spaces cannot account for the increase observed in the section area of the neural tube. As for the third possibility which relates to changes in the volume fraction of the cytoplasm in the neural tube it was found that it increases

with all of the LSD doses used (Fig. 2E). Therefore, the increase in the absolute volume of the cytoplasm in the neural tube turns out to be all the more important in view of the fact that the section area of the neural tube itself is enlarged under exposure to LSD at concentration of 12.5 and 50 μg/ml (Fig. 2A). Finally a swelling of the nuclei cannot account for the increased volume of the cytoplasm since the volume fraction of nuclei in the neural tube is stable (Fig. 2D). In fact, this last observation indicates that the absolute volume of the nuclei is reduced since the section area of the neural tube increases with concentrations of LSD of 12.5 and 50 μg/ml.

Organelles such as the mitochondria or endoplasmic reticulum cannot account for the increased volume of the cytoplasm: the volume fraction of mitochondria in the cytoplasm decreases (Fig. 4), while their surface to volume ratio is not modified. Both observations are taken as indications that the absolute volume of mitochondria may very well be unchanged in a cytoplasm of greater volume. As for the endoplasmic reticulum, its curve (Fig. 4) suggests that for concentrations of 12.5 and 50 μg/ml the volume of this organelle is, in absolute terms, decreasing in a swelling cytoplasm.

To recapitulate then, under the influnce of LSD the volume of the cytoplasm increases. Due to this increase there is (*1*) a reduction in the relative volume of the intercellular spaces and (*2*) an increase in the total section area of the neural tube. Presently, we do not know if these effects are reversible.

Our study rules out the possibility that nuclei, mitochondria or endoplasmic reticulum might intervene significantly in the swelling of the cytoplasm. One is tempted to raise the possibility that the drug, through interference at the cell membrane level, could cause abnormal distribution of water, as was described by Jurand and Tuft [24] in chick embryos exposed to β-mercaptoethanol. Yet, if such was the case the water imbalance, contrary to what was observed by Jurand and Tuft, would be such that it would not prevent the normal occurrence of neurulation. Thus we are undertaking a series of experiments to evaluate the effect of LSD on the surface membrane of cells.

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