# ELECTRON-MICROSCOPIC INVESTIGATION OF THE EFFECT OF NEUTRAL RED ON THE INTESTINAL EPITHELIAL CELLS OF PARROTS (MELOPSITTACUS UNDULATUS)

by

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## Introduction

In our earlier experiments we have investigated by means of the light microscope the changes occurring under the effect of neutral red in the duodenal epithelial cells of the parrot. Some hours after the administration of the dye we found in the apical part of the epithelial cells granules with high RNA content (C h l o p i n's crinom granules), whose number and distribution corresponded to the number and arrangement of bodies containing hydrolitic enzymes and PAS-positive lipids (lysosomes) as observed in the control. Under the effect of the dye acid phosphatase and esterase activity of the lysosomes decreased and the reaction assumed a diffuse character. At the same time the content in PAS-positive substance of the crinom granules increased. Our observations confirm the result of electron-microscopic investigations by other authors, who attribute a role to the lysosomes in accumulating foreign substances e.g. neutral red, that enter the cells (B y r n e 1964, K o e n i g 1963, K o v á c s – P é c z e l y 1965, T a n a k a 1962).

To clarify certain details of this process, it proved useful to investigate the manner of dye uptake and cytoplasmic changes with the electron microscope, and to compare the results with histochemical findings. The literature refers first of all to crinom formation which takes place in the cells with high ergastoplasm content of protein producing organs (seminal vesicle, pancreas, liver, etc.) (K o v á c s J. – H a f i e k B. 1964, K o v á c s – P é c z e l y 1965, S c h m i d t 1958, 1962.) It was expected that the process which takes place in the intestinal cells is different from that in the aforesaid organs. S c h m i d t (1962) has studied the effect of various basic dyes on the duodenal epithelial cells of *Daphnia pulex*, *Triton alpestris* and *Mus musculus*, especially after prolonged dye treatments. It seems likely, however, that it is the initial stages that are of significance in the mechanism of this process. The aim of our experiments we to describe the changes that take place in a short period of time (some minutes, 1 to 2 hours) after the administration of the dye.

## Materials and Methods

For the experiments 75 mature female parrots *(Melopsittacus undulatus)* of 32—44 g weight were used.

55 animale were administered orally 1.5 ml of 0.4% neutral red in distilled-water. The animals were decapitated 10, 20 and 30 minutes, and 1, 2, 4, 8, 12 and 24 hours after the administration of the dye.

One group of controls was given 1.5 ml distilled water orally, and deca-pitated at the same intervals as the dye-treated animals. A few animals were decapitated without treatment.

In preliminary studies we compared the structure of various portions of the small intestine and/or the duodenum; since we found no substantial diffe-rence in the structure of the epithelium — being of primary importance for our observations — of the various intestinal portions, in the later experiments only the central part of the duodenal loop were studied.

The pieces of duodenum were fixed in 1.0% $OsO_4$ (pH 7.2) and embedded in araldite. For the electron microscopic demonstration of phosphatase activity in the lysosomes, we applied glutar-aldehyde fixation.

Sections were made with the Ardenne ultramicrotome and contrasted with Reynolds' lead-citrate (R e y n o l d s 1963). The preparations were studi-ed with a KEM I and an ELMI D 2 electron-microscope.

## Results

The light microscopic structure of the duodenum of the parrot has been described (K á r p á t i A. 1966). In this paper we give a description of the ultrastructure of the epithelium, and of the submicroscopic structures associat-ed with the response induced by the action of neutral red; only the epithelial cells covering the apical and the median part of the intestinal villi will be con-sidered.

The free surfaces of the intestinal epithelial cells are covered with a large number of microvilli. Under the light microscope the microvilli represent the brush border; their alkaline phosphatase activity is high and they contain a PAS-positive substance similar to that of the secretion product of goblet cells. With the electron microscope the terminal web under the microvilli can clearly be identified. The epithelial cells are closely connected. Junctional complexes, as described by F a r q u h a r and P a l a d e in the intestinal mucosa of the rat, appear between two adjacent cells, near the free surface of the cells, in the longitudinal section (F a r q u h a r, M. G. — P a l a d e, G. E. 1963).

In high power pictures (12 000×) the tonofibrils directed towards the desmosomes appear clearly. The lateral cell membranes often form interdigita-tions.

The ovoid nucleolus of the epithelial cells appears in the lower third of the cell. This is characteristic of bipolar cells. It is covered by a double nuclear membrane, on which occasional pores are conspicuous. Some parts of the chro-matin substance of a granular-filamentous structure adhere to the inner sur-face of the nuclear membrane. The structure of the nucleolus is compact with occasional electron dense parts in it.

Mitochondria are found in a large number in these cells; they are gathered mainly above the Golgi zone and in the basal cytoplasm. They are simplex as a rule, but bifurcate sometimes. Part of their cristae are at right angles to the longitudinal axis, part of them run paralell with it. In some cases, especially immediately after feedings the mitochondria bend in a semi-lunar manner and envelop the lipid droplets that appear in the cytoplasm in a large number.

The Golgi apparatus consists of a few lamellae and a group of vacuoles located near them.

The ground substance of the cytoplasm is made up of typical ergastoplasm only in the apical portion of the cells; the cisternae of the ergastoplasm are found mainly about the mitochondria. Free ribosomes are found in a large number at the basal part of the cells. It is assumed that their occurrence in masses accounts for the high basophilia that can be observed with the light-microscope in these parts of the cells.

In the apical cytoplasm, above the Golgi apparatus, electron dense bodies are visible even with moderate magnification. According to their structure they represent two types. One type includes circular or ovoid bodies with a matrix consisting of relatively homogenous electron dense substance. They are surrounded by a single membrane. Appearing in the same number and arrangement as these bodies, granules of acid phosphatase and esterase activity can be identified with the light microscope in the apical cytoplasm containing also PAS-positive lipids. On the basis of these observations the above mentioned bodies are considered to belong to the lysosomes. The other group of the electron dense bodies have varying, irregular shapes; there is often no membrane round them, or it is difficult to identify. By means of the Perls reaction we have demonstrated in the light microscope the presence of iron-containing granules showing the same arrangement and localisation as the bodies above; these granules are probably pigment granules formed from lysosomes. It is likely, too, that their high density observed by means of the electron-microscope is related to their iron content.

Under the effect of neutral red significant changes take place in the cytoplasm of the epithelial cells within a short time after the administration of the dye.

About 10 minutes after the administration of neutral red large numbers of multivesicular bodies, consisting of small vacuoles, appear in the apical portion of the cells. They are surrounded by a membrane which is uninterrupted as a rule, but may sometimes show a hole towards the cytoplasm (Figs. 1, 2 and 3). In addition, we found some pinocytotic vacuoles at the basis of the microvilli. It is conceivable that the multivesicular bodies are formed through pinocytosis, too; but they may come about also as a results of diffusion of neutral red into the network of endoplasmic reticulum and causing there a bubbling process which may in turn result in the separation of small vacuoles of the multiveiscular bodies, dense bodies of varying shape and composition are formed in the apical cytoplasm under the effect of the dye. They are surrounded by membraneous elements. They are probably areas of damaged ergastoplasm in different stages of sequestration (Fig. 4).

A gradual fusion of the multivesicular bodies and the lysosomes or pigment granules is often observed after longer treatment (1, 2 or 4 hours). Con-

sequently many large electron-dense bodies of heterogeneous structure are formed and fill up the apical part of the epithelial cells almost completely. The phases of the gradual fusion of the lysosomes and multivesicular bodies can clearly be distinquished. The first phase of fusion is represented by the bodies which are made up of two parts, with respect to their content, in accordance with the structure of the initial components (Figs. 5 and 6). At a later phase the membranes of the multivesicular bodies disappear and the vesicles are scattered in the electron-dense bodies. By this time the vesicles contain various fragments of injured ergastoplasm and occasionally mitochondria, too.

Later the content of the granules formed in this way is gradually decomposed. At the end of the degradation the cytoplasm is full of large dense bodies, containing vacuoles, myelin figures and amorphous osmiophilic substance. The myelin figures appear to be the dominant elements in the degrading bodies (Figs. 7, 8.)

A comparison of the light microscopic and small power electron-microscopic pictures indicates that the dense granules are identical with the granules; containing RNA and PAS-positive substance; that is they are the electron microscopical equivalents of the crinom granules seen in the light microscope. In the course of crinom formation the quantity of ergastoplasm within the cells decreases. On the other hand, many free ribosomes and helically arranged polyribosomes appear in the cells, having been separated from the membranes of the ergastoplasm. In the other components of the cytoplasm, however, no sign of injury can be demonstrated, at least not in the initial phase of the process. The nucleus and most of the mitochondria remain intact, the mitochondrial cristae show a regular arrangement.

## Discussion

Several observations have been published concerning the changes which take place in the course of neutral red uptake and accumulation. Experiments made with a variety of objects e.g. liver (C h l o p i n, N. G. 1927), pancreas (B y r n e, J. M. 1964, C h l o p i n, N. G. 1927), kidney (C h l o p i n, N. G. 1927, S c h m i d t, W. 1962), intestinal epithelium of the seminal vesicle (K o v á c s J. 1967, K o v á c s J. — H a f i e k B. 1964, K o v á c s J. — P é c z e l y P. 1965), of the oviduct (C h l o p i n, N. G. 1927), nerve ( K o e n i g , H. 1963) and various connective tissue cells (T a n a k a, H. 1962) have yielded identical result. Under the effect of the dye highly basophilic granules appear in certain parts of the cells; these granules may form in different ways, but on the basis of their ultrastructure they belong to the group of residual bodies whose characteristic elements are the myelin figures made up of lipoprotein lamellae (K á r p á t i 1966, K o v á c s — P é c z e l y 1965, N o v i k o f f 1963, T a n a k a 1962).

We are not aware of similar studies concerning digestive apparatus of birds. It appears from our observations that the appearance of a large number of multivesicular bodies and some pinocytotic vacuoles within some minutes after the administration of the dye is the most conspicuous phenomenon in the epithelial cells of this object. It is known that a number of substances enter the
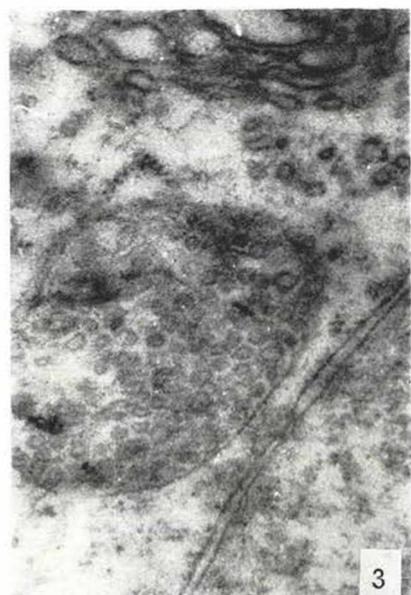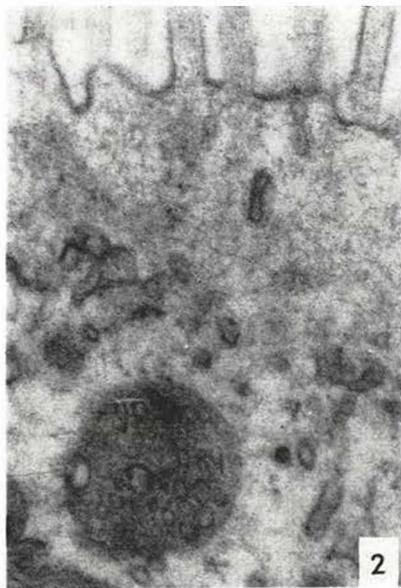
Figs. 1 — 2. Multivesicular bodies in the epithelial cells of the duodenum 4 hours
after the administration of neutral red. x 23 100 — 45 300

Fig. 3. Damaged parts of ergastoplasm and part of a lysosome in the epithelial
cells of the duodenum 4 hours after the administration of neutral red. x 35 600

Fig. 4. Part of the nucleus, a multivesicular body and helically arranged
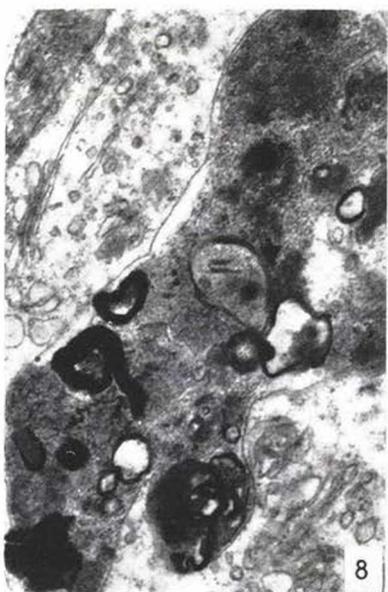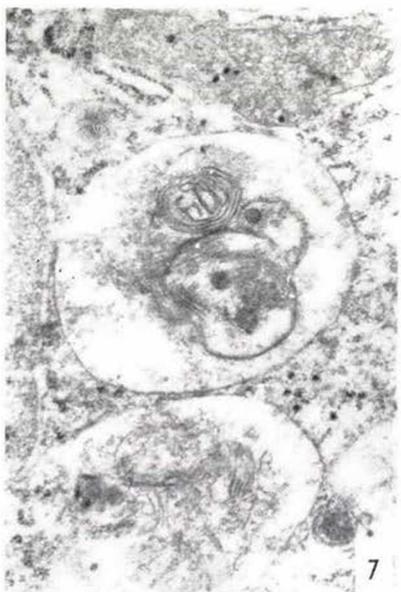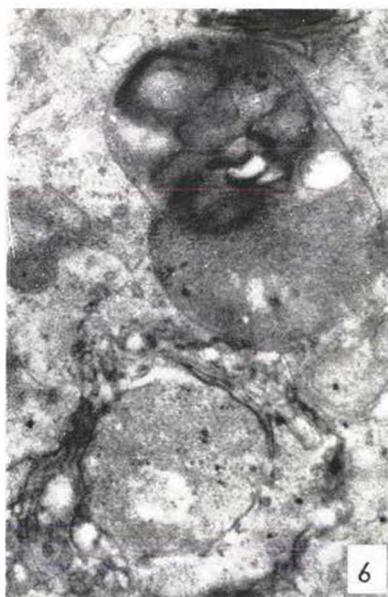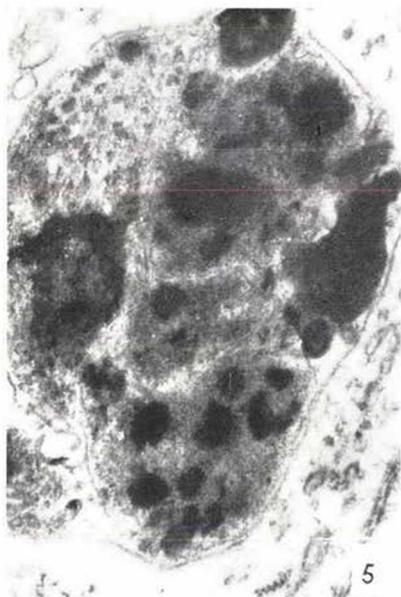polyribosomes 30 minutes after the administration of neutral red. x 21 000

Fig. 5. Multivesicular body and lysosomes fusing 2 hours after the administration of neutral red. x 30 800

Fig. 6. Crinom granules (with multivesicular bodies) in the epithelial cells of the duodenum 4 hours after the administration of neutral red. x 22 000

Fig. 7. Crinom granules with myelin figures 1 hour after the administration of neutral red. x 22 000

Fig. 8. Part of the epithelial cells of the duodenum 4 hours after the administration of neutral red. Note the large number of detached polyribosomes and crinom granula. x 16 800

cells by way of pinocytosis, so dye-accumulating vacuoles may form in this way (B e h n k e 1963, C l a r k 1959, N o v i k o f f 1961). The uptake of neutral red by pinocytosis has been observed in lymphocytes (T a n a k a 1962). Neutral red may have entered the cells through pinocytosis in our experiments as well; but it is likewise conseivable that the dye enters the endoplasmic reticulum by means of diffusion and that the vacuoles of the multivesicular bodies originate from its membranes by a bubbling process. Our observations show that the multivesicular bodies and the lysosome fuse, and that within the electron dense bodies (crinom granules) enlarged in this way, other cytoplasmic components appear as well later on. Fusion of the lysosomes and the pinocytotic vacuoles has been observed also by other investigators (C l a r k 1959).

It appears from studies on the cells of seminal vesicle that under the effect of dye sequestered fragments of ergastoplasm are converted into degrading bodies (K o v á c s 1967, K o v á c s — P é c z e l y 1965).

The initial phase of the process observed by us differ from the above findings substantially. One principal event of the mechanism of crinom formation is the fusion of the lysosomes with the multivesicular bodies that under the effect of the dye acid phosphatase and esterase activity of the lysosomes decreased and reaction assumes a diffuse character (K á r p á t i 1966, K o v á c s — H a f i e k 1964, K o v á c s — P é c z e l y 1965). Therefore it seems likely that the radical change occurring in the cytoplasm of intestinal cells after 1 − 2 hours of treatment are the consequence of leaking out of the lysosomal enzymes. They may cause the separation of ribosomes from the ergastoplasm membranes. The free polyribosomes were spread over the entire area of the cell after dye treatment. At the same time the number of ergastoplasmic cisternae decreased and they were gradually replaced by electrondense bodies (crinom granules).

At the final stages of this process all components of the crinom granules (multivesicular bodies, fragments of ergastoplasm and occasionally mitochondria) were degraded and as a result of this process numerous myelin figures appeared in the cells. Our results are very similar to those of B e h n k e, who observed degradation of damaged fragments of ergastoplasm and mitochondria and formation of lysosome-like inclusions from these degrading bodies in the duodenal epithelial cells of the rat embryo (B e h n k e 1963).

The circumstances of injury to cell organelles, of the fusion of injured plasm elements and the lysosomes require further studies of the experimental object (intestinal epithelium) used.

From a comparison of the literary data with our results of electron microscopic investigations it is clear that the changes which occur upon treatment with neutral red are based on different mechanisms. In cells with abundant ergastoplasm the crinom granules are formed through the sequestration of damaged ergastoplasm fragments. Our observations show that the process takes a different course in the epithelium of the intestine. Here the crinom granules are the result of a fusion between the lysosomes and the multivesicular bodies which have been produced by the treatment with neutral red; the damaged parts of the ergastoplasm play a minor role in the formation of the crinom granules.

### Электронно-микроскопическое исследование действия нейтрального красного на эпителиальные клетки двенадцатиперстной кишки попугая Melopsittacus undulatus

В апикальной плазме эпителиальных клеток двенадцатиперстной кишки животых наблюдались лизосомы, идентифицированные на основании их энзиматической (кислая фосфатаза и эстераза) активности.

10 мин. после введения нейтрального красного в апикальной плазме эпителиальных клеток появляются „мультивезикулярные тельца", состоящие из многочисленных маленких вакуолей, происходящих путём пиноцитоза или так, что краска, поступавшая в сеть эргастоплазмии, изолируется эргастоплазматическими мембранам.

Увеличив время действия нейтрального красного лизосомы набухают и часто связываются с мультивезикулярнами тельцами. Таким образом возникают большие гранулы (гранулы кринома) материя которых деградируется и в них появляются миелиновые структуры.

В эпителиальных клетках кишки гранулы кринома происходят главным образом путём слияния мультивезикулярных телец и лизосом в отличии от образования кринома в клетках поджелудочной железы и семенного пузырка в которых находится обильная эргастоплазма и которая и является источником этих гранул.

## Summary

Cytoplasmic changes upon treatment of intestinal epithelial cells of parrot with neutral red were studied.

In control animals many electron dense bodies (lysosomes) were found in the apical part of the epithelial cells covering the intestinal villi. About 10 minutes after the administration of neutral red, a large number of multivesicular bodies, made up of small vacuoles, appeared in the apical part of the cytoplasm. These were probably formed through pinocytosis. Another possibility is that neutral red entered the endoplasmic reticulum by way of diffusion and induced there the formation of vacuoles by bubbling of the ergastoplasmic membranes. In some cases the isolation of damaged parts of cytoplasm was found.

After prolonged treatment (2 – 4 hours) the lysosomes swelled and their fusion with the multivesicular bodies was often observed. Large granules were formed in this way; their substance was later degraded and myelin figures appeared as the end-products of decomposition. These bodies may be identified with the crinom granules visible in the light microscope.

We have found that in our experimental material the changes produced by the dye come about in a different way than in cells with well developed ergastoplasm, where the crinom granules are made up of decomposed ergastoplasm fragments. In the intestinal epithelium the formation of the crinom granules rests principially on the fusion of the lysosomes with the multivesicular bodies that have been formed in the course of the uptake of neutral red; the damaged cytoplasmic components appear in the crinom granules secondarily.

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