UPTAKE AND INTRACELLULAR DIGESTION OF PROTEIN (PEROXIDASE) IN PLANARIANS[1]

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It is thought (Willier *et al.*, 1925; Jennings, 1957; Rosenbaum and Rolon, 1960a) that digestion in aquatic planaria is exclusively intracellular, occurring in the spherules of the phagocytic gastrodermal cells. There is, however, very little information concerning the formation of the spherules, the rate of digestion of their contents, and their ultimate fate. Data based on the rate of disappearance of alkaline phosphatase from the spherules in planarians which had been fed raw earthworms (Osborne, 1955) were inconclusive, because of the impossibility of distinguishing exogenous from endogenous enzyme. Nor was it possible in these studies to determine whether the uptake of nutrients occurs exclusively by the phagocytic action of the gastrodermal cells. A new approach to these problems was suggested by the experiments of Straus (1959) on the intracellular disposition of parenterally administered horseradish peroxidase in the rat. Peroxidase, which does not occur in most cells of animal organisms, is readily visualized histochemically and can be used as a tracer for exogenous protein.

Materials and Methods

Specimens of *Dugesia tigrina* were starved for 10 days before the administration of peroxidase; this period of starvation is adequate to induce immediate feeding when food is offered but is not long enough to cause extensive resorption of the gastrodermal cells, which would delay the phagocytic uptake of food. Experiments were performed on both normal and pharyngectomized worms.

Pharyngeal feeding of peroxidase was carried out in the following manner, which simulates the conditions of normal feeding. The procedure was suggested by our observation that raw kidney is superior to other commonly-used foods for growth-promotion, and by the report of Straus (1959) that the greatest concentration of peroxidase, following parenteral administration in the rat, occurs in the kidney tubule cells. Mice were given an intracardiac injection of peroxidase (10 mg. in 1 ml. saline), and one hour allowed for glomerular filtration and tubular reabsorption of the enzyme (Straus, 1961). The mice were then killed by decapitation and the kidneys removed and frozen. Thin slices of kidney cortex were placed in jars containing starved planarians, and removed after the completion of feeding, which usually required about 30 minutes. At intervals ranging from 30 minutes to 8 days after the completion of feeding, planarians were removed and fixed for 12–24 hours in cold (4° C.) formol-calcium, rinsed for one hour in cold distilled water (4° C.) and embedded in 10% gelatin. The gelatin blocks were mounted on chilled cryostat

[1] Supported by NIH grants C-3996 and A-1699.

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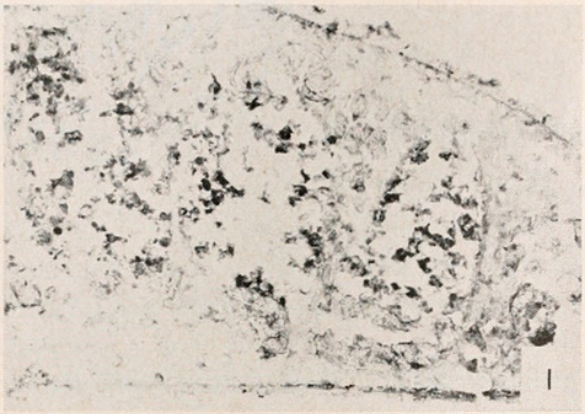
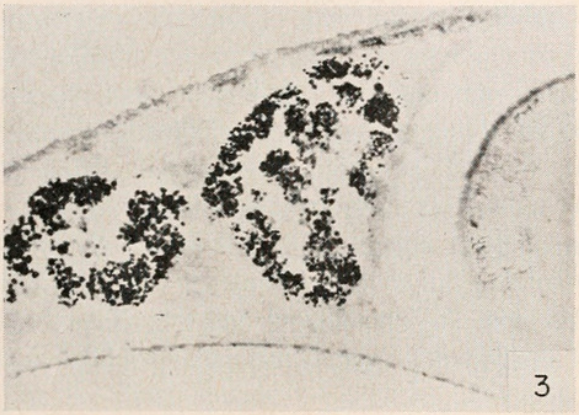
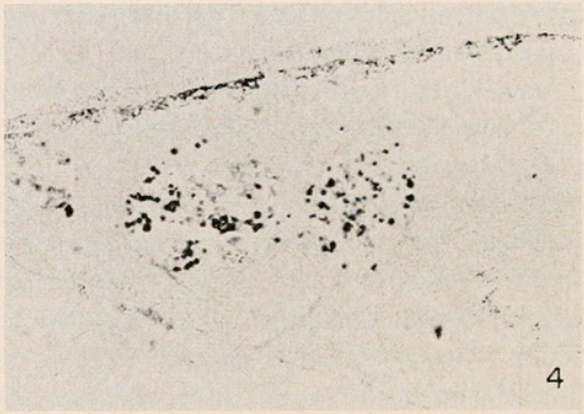
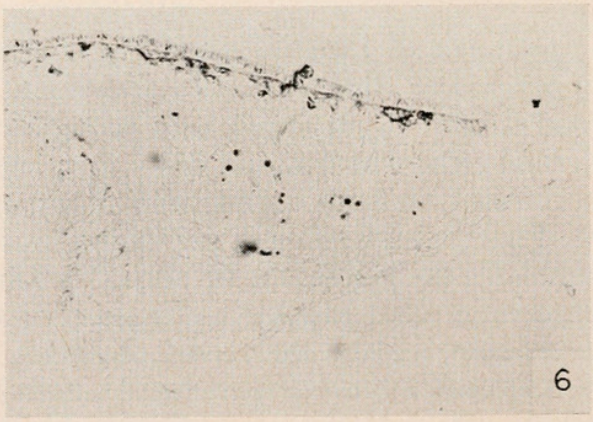








FIGURES 1–6. Stages in the accumulation and subsequent disappearance of peroxidase reaction product in the phagocytic cells.

FIGURE 1. Thirty minutes after feeding. Peroxidase activity is localized in diffuse form in the phagocytic cells. 75 ×.

FIGURE 2. Three hours after feeding. Peroxidase activity is predominantly concentrated in the forming spherules in the phagocytic cells. 75 ×.

FIGURE 3. One day after feeding. Peroxidase activity is present entirely within spherules of varying sizes. 75 ×.

FIGURE 4. Three days after feeding. Considerable digestion of the peroxidase has occurred. 75 ×.

FIGURE 5. Five days after feeding. Many of the spherules no longer show peroxidase activity. 75 ×.

FIGURE 6. Six days after feeding. Only an occasional spherule shows peroxidase activity. 75 ×.

object holders, frozen by contact with dry ice and cut at 8 μ in a Pearse cryostat. The sections were mounted on chilled slides without adhesive, air-dried for two hours, and the gelatin removed by gentle rinsing in a stream of warm water. The preparations were then incubated at 4° C. for three minutes in the medium recommended by Gomori (1952), as modified by Straus (1959), for the visualization of peroxidase activity. The incubation period was somewhat longer than that used by Straus, but this was necessary for sharp staining of the forming foodballs. Control sections were incubated in a similar manner with the omission of peroxide from the medium.

Permanent preservation of the blue color of the reaction product was achieved by complete dehydration of the specimens through absolute alcohol, followed by clearing in xylene and mounting in Permount.

In order to determine whether or not significant ingestion of protein can occur by extra-pharyngeal routes, planarians were pharyngectomized or transected at the base of the pharynx (which was removed) and the two halves separated from one another. One day was allowed for healing, since McWhinnie and Gleason (1957) have demonstrated that after this interval the cut surface of a transected planarian is covered with epidermis continuous with that of the rest of the organism. Specimens were then placed in solutions of horseradish peroxidase in filtered pond water, in concentrations of 10, 50 or 100 mg. per ml., for periods of 15 minutes to 6 hours. All worms exposed to peroxidase concentrations of 50 mg. per ml. for 6 hours and of 100 mg. per ml. for three hours died, but all survived exposure to 10 mg. peroxidase per ml. for 6 hours. The cause of death after prolonged exposure to higher concentrations of the enzyme is unknown. Control specimens were killed by fixation in cold formol-calcium before being placed in peroxidase-containing media.

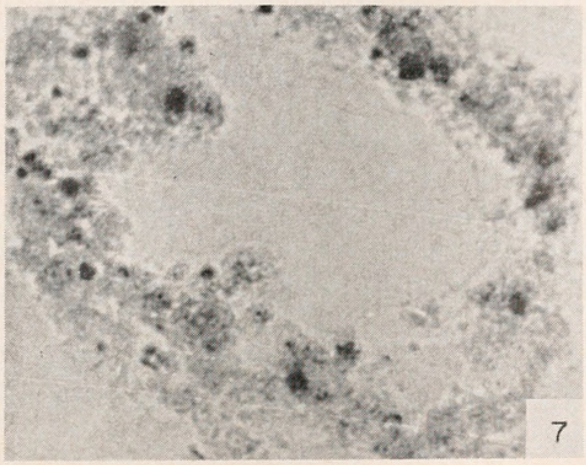
At the end of the exposure periods, the specimens were removed from the peroxidase solution, rinsed in filtered pond water for 5 minutes, and then chilled for a few minutes (4° C.) to produce a non-motile, slightly contracted condition. Cold formol-calcium was poured over each specimen individually, with care to ensure that each was fixed in a smooth, distended state. Fixation and subsequent treatment of these specimens were the same as for the worms fed mouse kidney.

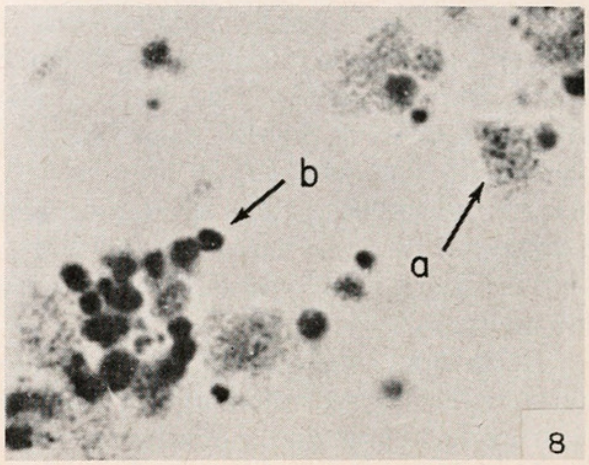
Results

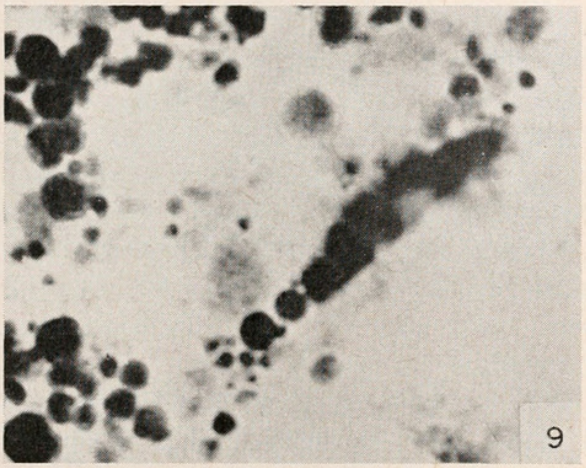
Pharyngeal feeding

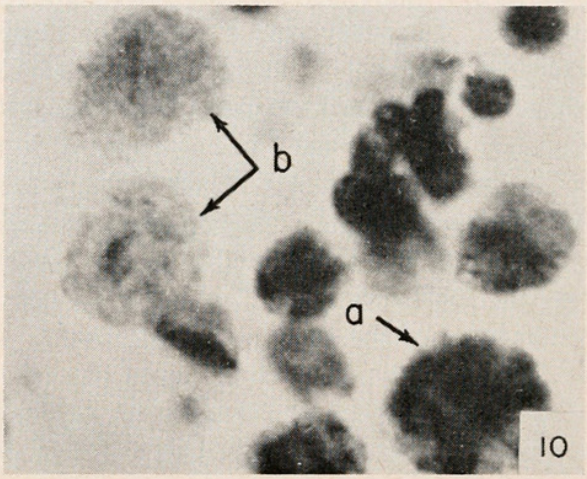
In planarians killed 30 minutes after the cessation of feeding there was a diffuse coloration of most of the phagocytic cells with the blue peroxidase reaction product (Fig. 1). Two to three hours following cessation of feeding, the reaction product formed discrete "droplets" within the phagocytic cells of the gut (Figs. 8, 9). Distinct spherules were present in specimens killed one day after feeding (Fig. 3), and gradual disappearance of enzyme activity occurred during subsequent days (Figs. 4–6). No activity was demonstrable on the eighth day following feeding.

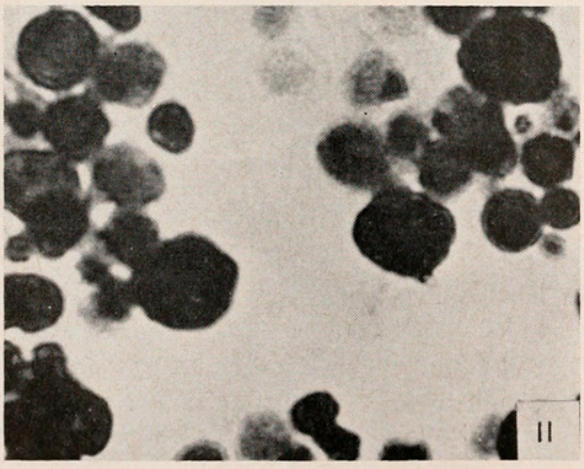
Details of the formation of intracellular protein spherules are shown in Figures 7–12. One hour after feeding, the peroxidase reaction product appeared in the form of small granules ranging in diameter from about 0.2 μ up to 2.0 μ (Fig. 7). They were present exclusively within the phagocytic cells; the larger granules appeared to result from the fusion of many smaller ones, since the number and the size of the granules within a cell varied inversely. They increased in size

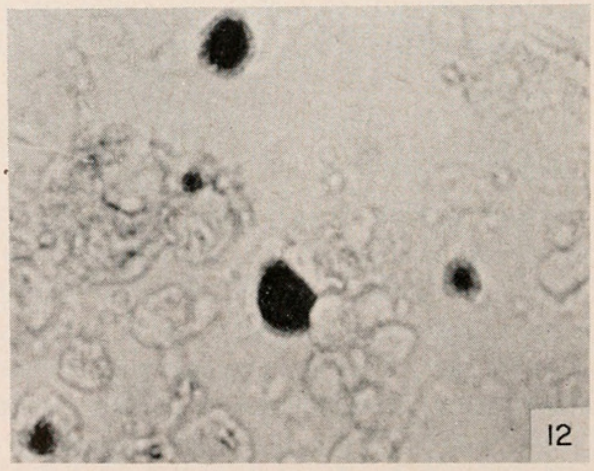












FIGURES 7–12. Stages in the formation of peroxidase-containing spherules.

FIGURE 7. One hour after feeding. Peroxidase reaction product appears as small granules ranging in size from the limit of visibility up to approximately 2 μ in diameter. 600 ×.

FIGURE 8. Two hours after feeding. Small granules are still present (arrow a), but the largest now approach the dimensions of spherules (arrow b). 600 ×.

FIGURE 9. Three hours after feeding. Note the phagocytic cell, cut longitudinally, containing numerous spherules and several smaller granules. 600 ×.

progressively (Figs. 8, 9), reaching a maximum diameter of 15–20 μ by the end of one day after feeding (Fig. 11). The classical intracellular "spherules" were clearly seen by the end of the third hour after feeding (Fig. 9). The onset of protein digestion could not be detected by the peroxidase method, but it was well-advanced by the second day after feeding, as indicated by the decrease in the numbers of spherules which gave an enzyme reaction. By the fifth day after feeding, the phagocytic cells were still filled with spherules, but only an occasional spherule contained active enzyme (Fig. 12). Three weeks after feeding, the phagocytic cells contained large numbers of refractile droplets similar to those previously (Osborne, 1955) shown by Nile blue and Sudan III staining to be fat. This suggests a conversion of protein to fat, as has been reported by Willier *et al.* (1925).

Extra-pharyngeal absorption of peroxidase.

When pharyngectomized planarians were exposed to media containing various concentrations of peroxidase, a definite penetration of the enzyme through the intact epidermis could be demonstrated (Figs. 13 and 14), and rows of peroxidase-positive granules were seen extending into the interior. Most of the penetration occurred through the ventral surface, but there was definite evidence of absorption through the dorsal surface as well. In Figure 13, penetration of peroxidase seems to be taking place via the canals left by the extrusion of rhabdites. A few phagocytic cells were filled with peroxidase-positive material within 30 minutes after the beginning of exposure (Fig. 15), and the apparent fusion of smaller into larger intracellular granules is illustrated in Figure 16. After 3 hours' exposure to peroxidase, the typical localization of peroxidase-positive material in the phagocytic cells of the gut was clearly demonstrable (Figs. 17 and 18), although the concentration of enzyme reaction product was very much less than in the case of intact planarians fed peroxidase-containing food.

Specimens killed by formol-calcium fixation prior to exposure showed minimal uptake of peroxidase and absence of intracellular localization.

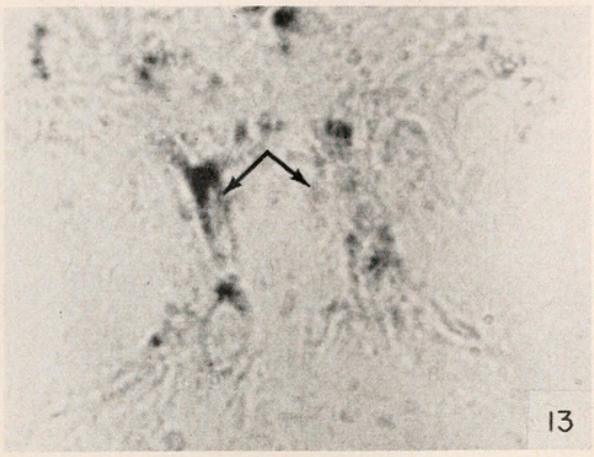
Discussion

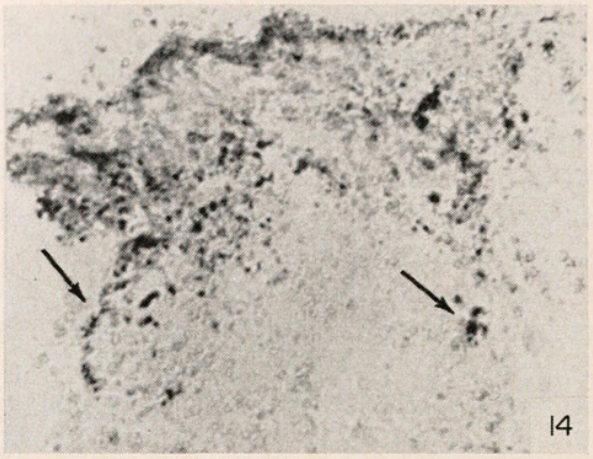
Studies on intracellular digestion in lower organisms are of interest both in their own right and for the light they may shed on similar processes in higher animals. Planarians are particularly well-suited for studies of this type because the phagocytic cells of the gastrodermis are readily accessible to exogenous materials, they respond to the uptake of these materials by the production of a variety of hydrolytic enzymes, and digestion is entirely intracelluar. While changes in enzyme activity associated with intracellular digestion are readily visualized by standard histochemical methods, changes in the materials undergoing digestion have been more difficult to demonstrate. The experiments of Straus

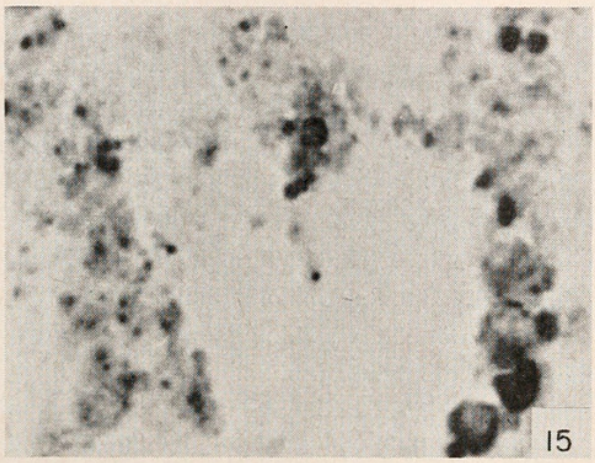
Figure 10. Three hours after feeding. Note the phagocytic cell, cut in cross-section (arrow a), containing a number of granules which appear to be coalescing, and other phagocytic cells (arrow b) which still contain many small granules. 1350 ×.

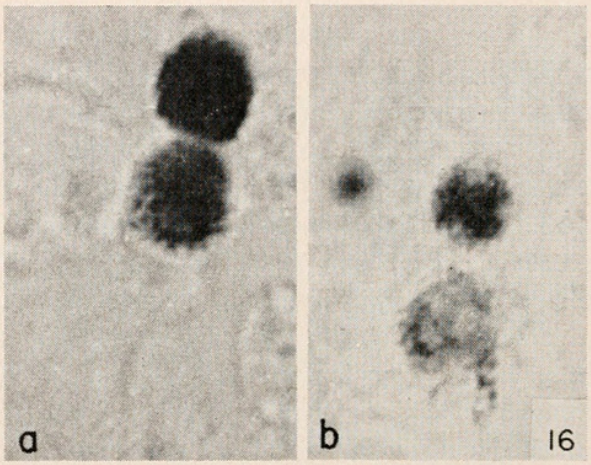
Figure 11. One day after feeding. Nearly all the peroxidase reaction product is present in well-formed spherules of varying sizes. 600 ×.

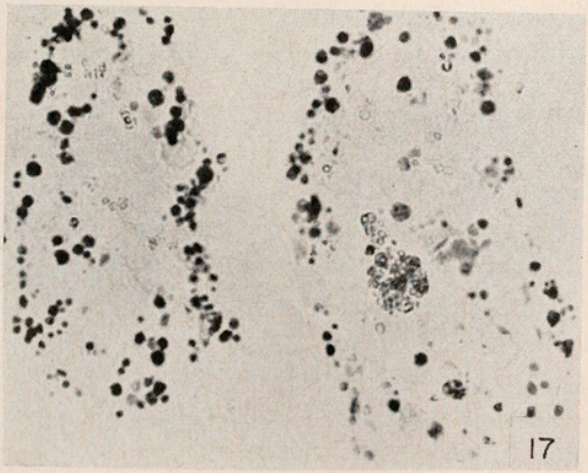
Figure 12. Five days after feeding. Large numbers of spherules are now devoid of peroxidase activity. 600 ×.











FIGURES 13–18. Transepidermal uptake of peroxidase in pharyngectomized planarians exposed to solutions of peroxidase in pond water.

FIGURE 13. Exposure time 15 minutes, peroxidase concentration 100 mg. per ml. Arrows point to concentration of peroxidase reaction product in canals from which rhabdites had been extruded. 1000 ×.

FIGURE 14. Exposure time 30 minutes, peroxidase concentration 50 mg. per ml. Arrows point to rows of peroxidase-positive granules extending from the surface toward the interior. 1000 ×.

FIGURE 15. Exposure time 30 minutes, peroxidase concentration 10 mg. per ml. Different stages in the formation of spherules are shown. 600 ×.

(1959) have shown that horseradish peroxidase is readily taken up by the cells of various tissues of the rat, and that its ultimate disposition can be followed by histochemical methods. This provides a convenient procedure which should be applicable to studies on the uptake and digestion of exogenous protein by the phagocytic cells of lower forms; the major uncertainty in the interpretation of the results concerns the degree of degradation of the enzyme molecule necessary for the abolition of its enzyme properties. It is interesting to note that the rate of disappearance of exogenous peroxidase activity from the phagocytic cells in the present study is in approximate agreement with the rate of disappearance of food spherules reported by Willier *et al.* (1925), based on non-specific histological staining methods.

The formation of intracellular food spherules appears to involve the progressive fusion of large numbers of very small granules. The smooth, circular profile of spherules of varying sizes suggests the presence of a limiting membrane of the type commonly associated with food vacuoles, a supposition which is supported by the presence of spherules devoid of peroxidase activity some days after the ingestion of peroxidase-containing food (Fig. 12). The mechanism of formation of these spherules is obscure and might profitably be studied by electron microscopy.

The transepidermal uptake of peroxidase in pharyngectomized planarians suggests a possible route by which nutrients are absorbed from the medium by planarians undergoing regeneration after transection or binary fission. Further work will be required before the quantitative importance of transepidermal absorption can be assessed.

While this material was being prepared for publication, our attention was drawn to the paper of Rosenbaum and Rolon (1960b), thus far available only in abstract form, in which the absorption by planarians of peroxidase dissolved in the medium was reported. Our results are in general agreement with theirs, although the procedure differed somewhat in the two experiments; the major difference in results is the much greater toxicity of peroxidase in our experience, the basis of which is to be investigated.

Summary

1. Stages in the ingestion of protein, and the formation and ultimate disappearance of spherules in the phagocytic cells of *Dugesia tigrina* were visualized histochemically by the peroxidase technique.

2. The formation of spherules involved the coalescence of numerous small peroxidase-positive granules. Typical spherules were present three hours after feeding kidney from a mouse previously injected with peroxidase, and maximal size of the spherules was achieved by the end of 24 hours.

FIGURE 16. Exposure time one hour, peroxidase concentration 100 mg. per ml. Earlier (b) and later (a) stages in the aggregation of peroxidase-positive granules in the phagocytic cells. Other phagocytic cells, as yet devoid of enzyme activity, are also present. 1350 ×.

FIGURE 17. Exposure time three hours, peroxidase concentration 50 mg. per ml. Typical spherules are present in the phagocytic cells. 300 ×.

FIGURE 18. Exposure time three hours, peroxidase concentration 50 mg. per ml. The enzyme activity is localized in the phagocytic cells but in much smaller amounts than after pharyngeal ingestion of peroxidase-containing mouse liver (*cf.* Fig. 2). 75 ×.

3. Peroxidase-positive material was confined to the phagocytic cells and disappeared gradually until none remained 8 days following feeding.

4. Pharyngectomized planarians exposed to a medium containing peroxidase in solution absorbed the protein through the epidermis, and formed typical spherules in the phagocytic cells. It is suggested that this may indicate a role of transepidermal absorption of nutrients in regenerating planarians.

LITERATURE CITED

GOMORI, G., 1952. Microscopic Histochemistry. Chicago, The University of Chicago Press.

JENNINGS, J. B., 1957. Studies on feeding, digestion and food storage in free-living flatworms (Platyhelminthes: Turbellaria). *Biol. Bull.*, **112**: 63–80.

MCWHINNIE, M. A., AND M. M. GLEASON, 1957. Histological changes in regenerating pieces of *Dugesia dorotocephala* treated with colchicine. *Biol. Bull.*, **112**: 371–376.

OSBORNE, PAUL J., 1955. Studies on phosphatases and lipases in certain Turbellaria. Unpublished doctoral dissertation, University of Florida.

ROSENBAUM, R. M., AND C. I. ROLON, 1960a. Intracellular digestion and hydrolytic enzymes in the phagocytes of planarians. *Biol. Bull.*, **118**: 315–323.

ROSENBAUM, R. M., AND C. I. ROLON, 1960b. Pinocytosis in phagocytes of planarians. *Anat. Rec.*, **137**: 389.

STRAUS, W., 1959. Rapid cytochemical identification of phagosomes in various tissues of the rat and their differentiation from mitochondria by the peroxidase method. *J. Biophys. Biochem. Cytol.*, **5**: 193–204.

STRAUS, W., 1961. Cytochemical observations on the transport of intravenously injected horseradish peroxidase and the development of phagosomes in the cells of the kidney of the rat. *Exp. Cell Res.*, **22**: 282–291.

WILLIER, B. H., L. H. HYMAN AND S. A. RIFENBURGH, 1925. A histochemical study of intracellular digestion in triclad flatworms. *J. Morph.*, **40**: 299–340.