CELLULAR MECHANISMS OF PROTEIN METABOLISM IN THE NEPHRON*

II. The Histochemical Characteristics of Protein Absorption Droplets

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(Received for publication, January 7, 1954)

We have shown (1) that the droplets forming in the epithelial cells of the proximal convolution of the rat kidney after the administration of various proteins give a strongly positive supravital reaction with dilute Janus green. This observation indicates that they are not simple accumulations of the absorbed material and suggests that they are complex objects composed of a mixture of the absorbed protein and mitochondrial elements, the latter derived from the rodlets of the renal cells which were observed to disappear concomitantly with the formation of the droplets. From such a hypothesis it would follow that the enzymatic content of the mitochondria is thus brought into immediate contact with the substrate, absorbed protein, which is ultimately to be digested inside the cell. The prompt reduction of the dye within the droplet supported this assumption.

The basis of these inferences is a staining reaction with a dye and its subsequent reduction in the droplet, and since the chemical or physical mechanisms involved in the reaction are unknown, more information is needed. It should, for example, be possible to demonstrate in the droplet those chemical substances which occur in the mitochondrial rodlets; the present study is an attempt to do so by means of the methods of histochemistry. The rigorous validity of many of these methods is open to question; some of them might better be termed more or less specific "staining reactions;" but these and other shortcomings have been thoroughly considered by others and are generally recognized. Our

* This work was supported by grants-in-aid from the Commonwealth Fund, the Life Insurance Medical Research Fund, and the New York Heart Association. A portion of the research was carried out at Brookhaven National Laboratory, Brookhaven, Long Island, under the auspices of the United States Atomic Energy Commission.

findings in the present study will be checked in later publications by biochemical and enzymatic procedures. Their immediate value lies in the possibility that the localization of substances can be cytologically visualized, provided diffusion or adsorption has not shifted constituents, and that this will afford evidence of a different sort to amplify that obtained by the more conventional techniques. Although these too are open to some criticism, they afford at least a departure point for the histochemical examination of the droplet, indicating as they do, that if it contains mitochondrial elements, then considerable amounts of phospholipid and some pentose nucleic acid should be demonstrable as well as absorbed protein.

The Fixation Reaction of the Droplets

The fixation characteristics of the droplets afford some information as to their chemical nature. Our original method was the use of Helly's solution or 10 per cent formalin and in most instances these procedures were adequate. It was observed, however, that occasionally the droplets were so poorly preserved, especially those following the injection of other proteins than egg white, as to be barely visible. As will appear later, this failure seems to be the result of a loss of lipids and was prevented, following Baker's suggestion (2), by adding calcium to the formalin solution. Carnoy's fluid, alcohol, or acetone dissolved the droplets completely so that in the stained sections the cells appeared empty (Fig. 1). The mitochondrial rods reacted similarly to all these fixatives.

For the Altmann-Gersh freeze-dry method of fixation tissues were immediately quenched in isopentane cooled with liquid N to −170°C. followed by dehydration in the model FD-11 unit of the Scientific Specialties Corporation. Paraffin sections were stained with iron-hematoxylin. The general appearance of the rodlets and droplets is similar to what is seen after fixation in Helly's solution (1), except that the former, though deeply stained, appear more delicate and the droplets are less heavily tinged (Fig. 2). The sections in our procedure passed through xylol and alcohol before being placed in the mordant of iron alum; as they had received no preliminary chromatization a relative solubility of lipoids may have been responsible for the attenuated appearance of both rods and droplets. Another striking difference in the general appearance of tubule cross-sections in freeze-dry preparations from what is seen after chemical fixation has been noted by Sjöstrand (3) and Bell (4); the proximal convolutions at first glance appear to have no lumen while in collecting tubules this space is quite apparent; but on closer inspection a lumen filled with a clear, faintly stained homogeneous substance is, however, to be seen. Bell speculates on the significance of this finding but does not mention the possibility that the homogeneous material may be the protein which is present in the tubule fluid of the normal rat. In our experiment there was in addition a considerable amount of egg white protein.

The Significance of the Gram Positivity of the Droplets

An extensive critical review of the mechanisms and meaning of Gram positivity has recently been made by Bartholomew and Mittwer (5). These authors conclude that the Gram-positive state cannot be ascribed to any unique chemical compound or complex. The removal of many substances, such as polysaccharides, phospholipids, and pentose nucleic acid, from bacteria by chemical extraction or enzymatic reaction causes Gram-positive organisms to stain negatively; the last two are of especial interest to us as they are mitochondrial constituents. However, Gram-negative organisms also contain PNA and other evidence suggests that physical factors, in particular the permeability of the stained object, are important to the course of the reaction. The authors also emphasize that Gram positivity is a matter of degree; the dye can be extracted from all bacteria, and standard procedures are therefore essentially for comparative results.

In our preliminary report (6) we have shown that the Gram positivity of droplets following egg white injection can be reduced by the action of crystalline ribonuclease and then renewed by "plating" the droplets with magnesium ribonucleate (6, Fig. 15).

The same experiment was repeated with the other proteins of our series, rat serum, horse serum, crystallized ovalbumin, and bovine serum albumin. Human hemoglobin was not used although, as we have shown, the droplets formed after its injection are Gram-positive; for hemoglobin itself is somewhat Gram-positive as can be seen in the red blood cells of histological sections, a coincidence which would confuse the interpretation of the experimental results.

The sections were prepared from the kidneys of sets of animals injected intraperitoneally by the standard procedure previously described (1) that gives a maximum of droplet formation, and fixed in 10 per cent calcium-formalin solution. Pairs of slides were run together for 1 to 3 hours, one in distilled water, the other in an aqueous solution of protease-free crystalline ribonuclease (0.2 mg./1 cc.) adjusted with 0.01 N NaOH to a pH of 6.0 to 7.0 at 37°C. The sections were then stained by our standard Gram procedure: 30 minutes in saturated alcoholic crystal violet, 2 minutes in Lugol's solution, and decolorized in a 1 to 3 xylol-aniline oil mixture.

The control slides which had been in distilled H_2O at 37°C. showed deeply stained Gram-positive droplets; those which had been subjected to the action of the enzyme were filled with droplets which varied in appearance. Some might be considered still Gram-positive but others were so faintly and irregularly tinged as to appear like eroded remnants of the original solid droplets. Similar incomplete removal of stainable material by ribonuclease in other experiments (7) has been interpreted as an indication of the presence of a protein residue; this conclusion would seem applicable in the case of the droplets. The fact remains that something was removed by the enzyme, as indicated by the altered staining reaction of the droplets. Placing the poorly stained extracted sections in a weak solution of magnesium ribonucleate for 15 minutes, rinsing

thoroughly, and restaining by the Gram procedure resulted in strongly positive droplets. This positivity was however less resistant to decolorization than that of the original untreated droplets for even moderately prolonged differentiation removed most of the dye.

Lipids have been found to be responsible, in certain experiments, for the Gram reaction, and analyses of mitochondria have shown them to be rich in these substances. Extraction in pyridine at 60°C. for 24 hours had no effect on the Gram positivity of egg white protein droplets in sections from calcium-formalin fixed material (Figs. 3 and 4). Since it cannot be assumed that all lipid was completely extracted from the calcium-formal fixed tissue (2) and, as later experiments will show, its complete removal results in a disintegration and disappearance of the droplets, there seems to be no way to test directly what part, if any, lipid content may contribute to the Gram positivity of the droplets.

Finally, as we have previously reported (6, 1), under no conditions did the mitochondrial rodlets stain with the Gram procedure, yet analyses have shown that these structures contain both PNA and phospholipid.

To summarize, the intense Gram positivity of the droplets cannot be ascribed to any specific chemical constituent; there is tentative histochemical evidence that PNA, phospholipid, and protein all may contribute to the reaction. Judging from the ubiquitous distribution of the latter and its usual lack of reaction to Gram staining, the protein seems not likely to be important. Most interesting is the fact that the mitochondrial rodlets, known to contain these same substances, are entirely negative. This contrast would seem to indicate physical differences in the organization of the constituent substances in the two cases and it may be that therein lies the cause for the distinctive Gram positivity of the droplets.

The Pyronin–Methyl Green Reaction of the Droplets

Various modifications of Unna's method of staining with pyronin and methyl green have been extensively used to identify the nucleic acids of cytological material, the former staining cytoplasmic pentose nucleic acid red and the latter the desoxyribose nucleic acid compounds of the nucleus bluish green. Considerable difference of opinion is noted among current investigators as to the specificity of the stain and it has been shown that the purity of the dye, the manner of tissue fixation, and the nature of the staining procedure all may affect the end-result of the reaction. These problems have been fully considered by Taft (7), and our method of staining is based, with some modification, on his standard procedure (8).

Tissue from kidneys containing droplets after the standard procedure of intraperitoneal injection of the various proteins was fixed in 10 per cent formalin, in Helly, and in Giemsa solution. The first and third of these fixatives gave somewhat better results than the bichromate mixture, for with the latter the red-pink of the pyronin was less bright and clear. Paraf-

fin sections were stained for 3 minutes with a solution consisting of 100 cc. of 0.5 per cent methyl green, purified as Taft describes by extraction with chloroform, with M/10 acetate buffer at pH 6.0 in which 0.2 gm. pyronin B had been dissolved. Rinsed and blotted, they were then differentiated in a mixture of 3 parts tertiary butyl alcohol and 1 part absolute ethyl alcohol.

The droplets after the injection of all the proteins (rat serum, horse serum, crystalline ovalbumin, bovine albumin, egg white proteins, and human hemoglobin) were pyronin-positive, appearing as deep rose-pink objects in a cytoplasm which was also faintly pink. The rodlets in cross-sections of convolutions that contained no or few droplets were not much more deeply stained than the surrounding cytoplasm and, though they were visible as striations, did not therefore stand out as do the contrasting clear cut rods of a preparation stained with iron-hematoxylin. The chromatin of the nuclei of the renal cells was a blue-green (Figs. 5 to 7).

The color reactions of the sections were therefore such as might be interpreted as evidence of the presence of DNA in nuclear chromatin and PNA in cytoplasmic droplets. It is when this conclusion was tested by extraction procedures that, as in the experience of past investigators (7), difficulties appeared. In our experiments, crystalline ribonuclease used as previously described and also extraction for 30 minutes in 5 per cent trichloracetic acid at 90°C., failed to completely prevent a faint pinkish coloration of droplets when the sections were later stained with the pyronin–methyl green mixture, although the cytoplasm about the droplets was practically colorless and the nuclei, after the extraction in hot trichloracetic acid, did not stain with methyl green.

The Absorption of Ultraviolet Radiation (2537 A) by Droplets and Mitochondrial Rodlets

With the thought that photomicrography at 2537 A might afford further evidence in regard to the distribution of the nucleic acids in the renal cells, sections of kidneys fixed in 10 per cent formalin from animals injected with egg white proteins were mounted on quartz slides. The final preparation of the sections, including the extractions with ribonuclease and trichloracetic acid and the photographic procedures were done by one of us (M.J.M.) at Brookhaven National Laboratory.

Energy at 2537 A was isolated from a Hanovia SC 2537 low pressure mercury discharge lamp by means of two 60° quartz prisms. A conventional photomicrographic apparatus employing Cooke, Troughton, and Simms quartz optical elements was set up with the mercury source and arranged for Köhler illumination. Kidney sections on quartz slides were mounted in glycerin and chosen, mapped areas were photographed before any and after each successive treatment. Digestion with crystalline ribonuclease (protease-free), and extraction with hot TCA, were carried out as described above.

While considerable effort was made to keep illumination and other photomicrographic conditions constant between treatments, some small variations were unavoidable. Hence the resulting photographic series, while permitting precise localization of significantly absorbing

materials, at best reflect only in a gross way changes in amount of light absorption, and thus of absorbing materials, after treatment. A more quantitative interpretation must await microphotometric analysis.

Figs. 8 to 10 show the absorption pattern in the same two unstained cross-sections of a proximal convolution from a normal rat which had received no injection: Fig. 8, the untreated section; Fig. 9, after the action of ribonuclease; and Fig. 10, after extraction with hot 5 per cent trichloracetic acid. It will be observed in Fig. 8 that a darker band of cytoplasm, which is faintly striated, occupies the position of the palisade of mitochondrial rods; the lighter band contiguous with the lumen of the tubule is not the brush border of the renal cells but the free apical cytoplasmic zone into which the mitochondrial rods do not penetrate. After the action of ribonuclease this separation of the cell into two strata is less distinct, and is barely appreciable in Fig. 10 after hot trichloracetic acid had removed all nucleic acids including the DNA of the nuclei.

Figs. 11 to 13 are similarly prepared ultraviolet photomicrographs of the same two cross-sections of a proximal convolution from the kidney of a rat which had received a standard intraperitoneal injection of egg white proteins. The greatly swollen epithelial cells in Fig. 11 are filled with droplets which appear clearly as ring forms due to their refractivity. There is a considerable and irregular degree of apparent diffuse absorption in the cytoplasm in which the droplets lie, an observation which recalls the appearance of similar regions previously noted in sections stained with iron-hematoxylin (Figs. 9, 11, 20 in Paper I (1)) where droplets were surrounded with a diffuse basophilic substance which appeared to have its origin in disintegrating rodlets. Since there is little change in appearance after the action of the ribonuclease (Fig. 12), or after hot trichloracetic acid (Fig. 13), except that in the latter instance the DNA of the nuclei has been removed, it may be assumed that the blotches of persisting cytoplasmic absorption are due either to high local concentrations of protein or to refraction by tightly packed masses of droplets, and possibly to both.

The results of these experiments resemble those obtained by the pyronin–methyl green stain. Both mitochondrial rodlets and droplets are similar in that they do not absorb sufficiently to register as positively as would be expected if they were rich in PNA. If they contain this substance, it is therefore in such small amount as to be beyond the limits of appreciation by a method which is adequate for the demonstration of the DNA content of the nucleus.

The Reaction of the Droplets to the Acid Hematin-Pyridine Extraction (Baker) Procedure for Phospholipids

The Baker (2) stain for phospholipids is of particular value to this study not only because of the extensive tests of its specificity to which both its originator

and Cain (9) have subjected it, but also because among the substances tested and found to give a negative reaction are several which were used in the production of the droplets in our experiments, for example egg white, blood serum, and blood albumin. Moreover, the reaction has been shown by Baker to be strongly positive for the rodlets of the mouse kidney, the mitochondrial apparatus so rich in phospholipids which according to our hypothesis furnishes by its dissolution an important constituent of the droplet. The procedure also has a double check on the validity of its result: although the phospholipids react with a deep blue-black that usually is sufficient to distinguish them from the muddy browns of other cellular constituents, such as proteins, extraction with pyridine, by removing them completely, allows a resolution of any uncertainty.

Tissues from animals injected intraperitoneally by the standard procedure with all the proteins were fixed in Baker's formol-calcium solution and weak Bouin's fluid. As the author emphasizes, it is essential to follow exactly his stated concentrations, temperature and time periods; the reader is referred to the original publication for these details. In brief, the tissues were fixed in 10 per cent formol-calcium solution or Bouin's fluid, transferred to 5 per cent bichromate-calcium solution at 60°C. for 24 hours, washed, and embedded in gelatin. Sections were placed in bichromate solution, washed, and then stained for 5 hours at 37°C. in acid-hematin solution, differentiated for 18 hours at 37°C. in borax-ferrocyanide solution, washed, and mounted in glycerin jelly. For the extraction of the phospholipids, the Bouin's fixed tissue was run through alcohols and placed in pyridine for 24 hours at 60°C.; the further treatment with bichromate, embedding, and staining was that previously described.

The reaction of the droplets formed after the injection of all the proteins was similar; they were an intense deep blue-black. The mitochondrial rodlets in cross-sections of convolutions free of droplets were equally deeply stained (Figs. 14 to 16). Sections stained after extraction in hot pyridine for 24 hours showed neither droplets nor rodlets (Fig. 17). The one exception, and this was to be expected from Baker's examination of the reaction, was that after pyridine extraction the hemoglobin droplets, though disintegrated as a result of the removal of their lipids, left a residue of blackish discoloration in the cytoplasm. The observation is of some interest indicating as it does that the droplet form of the absorbed protein is dependent, not on its protein but on its lipid content, a conclusion the importance of which will appear in the final paragraph of our discussion.

An interesting difference was noted in some of the preparations between the staining of the mitochondrial rodlets and the droplets. Owing apparently to irregularity in penetration of the constituents of the fixing reagents, a peripheral zone several cross-sections of tubule thick extending around the edges of the section showed the usual picture of black rodlets and droplets, whereas deeper in the tissues only the droplets were stained. The impression is thereby given that the phospholipids of the droplets are in some other form or combination than was the case in their original situation in the rodlets. There was

no difference in the extractability by pyridine in these tissues; throughout the section the droplets were no longer visible.

Variation in the Histochemically Demonstrable Components of the Droplets during Their Formation and Autolytic Disappearance

As we have previously noted (1), egg white droplets begin to appear in visible form when stained by the Gram method about 8 hours after the injection of the protein. They are then to be seen as faint rose-pink objects when stained with pyronin–methyl green mixture. When the Baker acid–hematin method for phospholipids was used on the same tissues, definitely stained droplets could be observed 6 hours after injection and in some instances even at 4 hours. Before they reached their maximum development at 18 hours, those of a single tubule cross-section varied individually in the intensity of their reaction with each of the three methods, an irregularity that disappeared as the maximum was approached, the cells then becoming filled with evenly and strongly reacting droplets.

In experiments in which, on the contrary, the egg white protein droplets were disappearing during the first few days after the injection, a similar irregularity of staining reaction developed. The droplets that persisted still retained their original reaction to the three methods, however, and during autolysis the same irregularities were noted before their final dissolution.

There is evidence, therefore, that the phospholipid content of the droplets is acquired early in their formation and that their constitution changes either chemically or physically not only as they mature to large strongly reacting droplets but in their final metabolic disposal by the renal cells.

The Demonstration of Absorbed Protein in the Droplets

It is clear from our observations thus far reported that the various staining procedures for droplets depend not on any protein content they may have but on cytoplasmic constituents, such as phospholipid, which have been incorporated in them. The demonstration of the protein itself presents a peculiar combination of the obvious and the difficult. In the first category is the case of hemoglobin: although it is not true, as commonly stated, that one observes "droplets of hemoglobin" in the renal cells, the color in fresh material (Fig. 28 in Paper I (1)) indicates that the droplets contain a certain amount of this substance and staining of them in cells of fixed tissues by the benzidine reaction as well as the observation of the product of its digestion, hemosiderin, confirm its presence.

Another of the substances of our series that produces droplets, the mixture of egg white proteins, has distinctive histochemical characteristics, some 30 per cent of its protein content being the glycoprotein ovomucoid, a substance that can be demonstrated by the periodic acid–Schiff (PAS) reaction. In con-

trast, the glycoprotein content of horse serum is low and that of the preparations of bovine serum albumin and crystallized ovalbumin, possibly present as contamination, presumably still less. The large droplets in the cells of the proximal convolution that followed egg white injection were strongly PAS-positive, equally so as those structures of the kidney, basement membrane and brush border, which normally give the reaction (Fig. 18). The intensity of the reaction throughout these plump droplets suggests that their large size as compared with all the other protein droplets may be due to the high water content of the mucoid element in their constitution.

In the sections of proximal convolution that were filled with the smaller Gram-positive crystallized ovalbumin droplets a few were seen that stained (Fig. 19), but none so strongly as the brush border of the cells that contained them. Many of these cytoplasmic objects did not appear as discrete, rounded droplets but as irregular flecks of pinkish stain, apparently representing droplets with a minimal polysaccharide content that may have been a contaminant of the ovalbumin preparation. Sections of proximal convolution that had previously been seen filled with Gram-positive droplets following bovine serum albumin (Fig. 20), rat serum (Fig. 21), and horse serum, contained an even lesser number of pale pink droplets and flecks of color, these possibly a reaction to seromucoids of the rat serum or to contaminants of the preparation of bovine serum albumin.

The application of the PAS stain afforded additional information concerning the mechanism of the disposal of the protein droplets by the renal cells. It had been noted that though many egg white droplets disappeared from the cells of the living animal during the first few days following injection, some persisted for long periods; this was less true of blood protein droplets which disappeared more promptly. A similar difference was also observed between the manner of disappearance of the two sorts of droplets as a result of *in vitro* autolysis; rat serum droplets disintegrated promptly whereas egg white droplets were markedly resistant. Sections of the latter preparations stained by the Gram method after 48 hours of autolysis at room temperature showed cellular debris but no recognizable droplet forms (Fig. 43 in Paper I (1)). When sections from the same block were stained with the PAS procedure, ill defined rounded remnants of the droplets could still be seen in various stages of disintegration (Fig. 22). This observation suggests that it is the ovomucoid element in the egg white droplets that is responsible for their resistance to both autolysis *in vitro* and cellular disposal *in vivo*.

Currently available histochemical reactions for proteins depend on the demonstration of various amino acids which form part of the larger molecule and they are therefore not specific in any rigorous sense; moreover as has been observed, in histological preparations protein is ubiquitous. A strongly positive reaction localized clearly to a droplet that stands out sharply on the more feebly stained cytoplasm of the cell indicates only the presence of a consider-

able concentration of the amino acid, and it must be assumed that it is a part of the particular protein molecule the injection of which resulted in droplet formation. In our experiments Weiss, Psou, and Seligman's procedure for protein-bound NH_2 groups (10), Danielli's method for tyrosine, tryptophane, and histidine (11), and Barrnett's stain (12) for protein-bound sulfhydryl groups were used.

Sets of rats were injected intraperitoneally with the standard dosage of egg white, bovine serum albumin, rat serum, or horse serum; the tissue fixed in Giemsa and formalin solutions and the various procedures followed as described. In most sections irregularities were encountered in positive reactions. In few instances did all the droplets throughout the entire section react evenly and equally; in some preparations the tubule cross-sections lying at the edge of the tissue would be strongly positive while those lying deeper were negative. As the conditions of the reactions must in all cases be exceedingly complex, it is not surprising that optimum conditions, particularly during the period of the penetration of the fixing solution, are not uniformly obtained, so that it seemed permissible to disregard a negative reaction when strongly positive examples were found in the same preparation.

In using the Weiss-Psou-Seligman method for the demonstration of protein-bound amino groups, sections of Giemsa-fixed tissue were placed for 1 hour at 40°C. in a solution of 20 mg. of naphthaldehyde in 20 cc. of acetone. Washed free of reagent, the addition product was then coupled with tetrazotized diorthoanisidine to form a blue azo dye at the site of the reaction. The general cytoplasm and the nuclei in cross-sections of convoluted tubules reacted faintly with a pinkish mauve tinge; the droplets of egg white and of bovine albumin were a deep blue (Fig. 23).

The Danielli procedure gave the most clear cut and strongest reaction in the droplets. Sections were immersed for 15 minutes at 4°C. in freshly tetrazotized benzidine, washed in water in three changes of veronal buffer at pH. 9.2, then in K(H) acid in the same buffer for 15 minutes, washed again in water, dehydrated, and mounted in balsam. The cytoplasm of the cells of the proximal convolution reacted moderately with a light orange tinge: on this background the large egg white and smaller bovine albumin droplets stood out as deep reddish orange objects with occasional tinges of reddish purple (Figs. 24 and 25).

Our procedure in demonstrating protein-bound SH groups in formalin-fixed tissue followed that of Barrnett (12).

Deparaffinized sections from water were incubated 1 hour at 50°C. in a solution containing 35 cm. 0.1 M Michaelis barbital buffer (pH 8.5), 15 cc. absolute ethyl alcohol, and 25 mg. 2,2-dihydroxy-6,6′-dinaphthyl disulfide. After cooling and washing in distilled water acidified to pH 4 with acetic acid the excess reagent was removed by passage through graded alcohols, rinsed in distilled water and stained for 2 minutes in a freshly prepared solution of 50 mg. tetrazotized diorthoanisidine in 50 cc. 0.1 M Sörensen phosphate buffer at pH 7.4. The sections were then washed in running tap water, passed through alcohols, and mounted with clarite.

The color reaction varied with the concentration of SH groups from a pink through lavender to blue. The cytoplasm of the proximal convolutions reacted with the development of a lavender tinge which, as Barrnett has described, is definitely stronger than that of other tubules in the medulla. The mitochondrial rodlets were clearly evident as lavender-blue striations. The droplets of egg white and bovine albumin were much more on the blue side of the color range, either a lavender-blue or, in some instances, a clear, dark blue (Fig. 26).

In summary, it can be said that all the methods showed much higher concentrations of various protein-bound amino groups in the droplets than in the general cytoplasm of the cells which contained them. This presumptive evidence of the protein content of the droplet is supported by the specific demonstration of one of the injected proteins, the glycoprotein of egg white.

DISCUSSION

The use of histochemical procedures to test our hypothesis that the droplets appearing in experimental proteinurias are composed of mitochondrial substances[1] and the absorbed protein has given results that are in some parts clear cut and comprehensible and in other parts inconclusive. Difficulties might indeed have been anticipated in the analysis of such a labile object as the droplet, for it is clear from our description of it that it is not a static entity but rather a locus of metabolic activity in which constantly shifting dynamic processes of metabolic synthesis and degradation are structurally expressed by its formation, evolution, and disappearance. Neither its chemical nor physical constitution is therefore fixed or constant.

All investigators using every available method, both histochemical and biochemical, are agreed that a major constituent of the mitochondria is phospholipid. Equally definite and conclusive is our finding by the Baker acid–hematin–pyridine extraction procedure that a major component of the droplet is phospholipid. This observation supported as it is by the behavior of the droplet to various fixation and extraction procedures, proves that this intracellular structure is not, as has been previously assumed, a simple droplet of absorbed protein, and the nature of the cytoplasmic constituent observed, phospholipid, is strong presumptive evidence that this element is derived from the dissolution of the mitochondrial rodlets so rich in it, which was observed concurrently with the formation of the droplets. A further detail regarding the phospholipid of droplet and mitochondrium may be noted; the phospholipid of the former is

[1] Attempts to localize various enzymes (alkaline and acid phosphatase, lipase, cytochrome-C oxidase) in cytological structures (droplets and rodlets) were unsuccessful since the required fixation either did not preserve the structures or resulted in a diffusion of the enzyme throughout the cell and a resulting minute granular reaction that bore no relation to its cytological components. Possibly freeze-dry fixation might have been more successful, but it was concluded to leave the determination of the enzymatic content of the cell particulates to the more dependable techniques of biochemical procedure.

more "reactive" than that in the rodlet, an observation which suggests that its association with the absorbed protein is in some way different, whether physical or chemical, from that obtaining in the original mitochondrial substance.

It should be emphasized that our conception of the relation of rodlet to droplet is not that of the older literature whereby the mitochondrium is "transformed" into the new structure, nor is there evidence for the modern version that the droplet is the persisting mitochondrium on, or in which, some new element is adsorbed. The histological evidence suggests rather that the rodlets on dissolution, contribute their constituents to the cytoplasmic "pool," and that from this source the droplet is formed, with a constitution so like that of the original cell organelle that the specific supravital reaction with Janus green is similar.

Our search for the presence of some form of pentose nucleic acid in both droplet and mitochondria was less conclusive in its results. Although the majority opinion agrees that smaller cytoplasmic elements, the ill defined and invisible "microsomes," contain the bulk of it, biochemical analyses indicate that the mitochondria also contain a certain amount. Some investigators (13) have challenged this conclusion and claim that the small quantity found by chemical analysis is a contaminant of the mitochondrial preparation, resulting from adsorption occurring during the technical manipulation. In any case, by the most generous interpretation of current analyses, the ratio of PNA to phospholipid as a mitochondrial constituent would seem to be of the order of 1 to 10.

The conventional color scheme of the methyl green–pyronin stain of our preparations might seem at first glance to distinguish and identify clearly enough the blue-green nuclear DNA and the pink cytoplasmic PNA of rodlet and droplets. But rodlets, though visible as pink striations, do not stand out like the clear cut, deeply stained structures of the Baker phospholipid procedure, an observation which might be taken as evidence that they contain no more or even possibly less, than the general cytoplasm of the cell. The droplets derived from all sorts of protein are more "positive," *i.e.* a deeper pink, than the rodlet or cytoplasm. This fact may well be due to their relative large bulk or density rather than to a higher content of nucleic acid.

These assumptions as to the presence of a small amount of PNA in the droplet, which conform well enough with both our original hypothesis of its mitochondrial origin and with current biochemical analyses of mitochondria are, however, considerably weakened by the negative results of the extraction techniques; for neither specific crystalline ribonuclease nor trichloracetic acid completely removed all the pink staining material from all the droplets, an observation similar to that of other observers (14, 7) who have criticized the

specificity of the pyronin reaction for PNA in other tissues. Nor do the experiments with ultraviolet energy at 2537 Å resolve these uncertainties; the rods and droplets absorb poorly; they register as visible structures on the photographic plate but not as dark black objects comparable to the nuclei. As the nucleic acid concentration in the nucleus is some 15 times higher than that reported for the mitochondria this might be expected; the fact that extraction with ribonuclease and trichloracetic acid did not appreciably lessen their ability to absorb is also to be expected. However, as in the case of the methyl green–pyronin reaction, we are still left with some uncertainty about the presence or absence of small amounts of PNA in the rods and droplets.

The Gram positivity of the droplets is reduced if not entirely removed by extraction of nucleic acid with enzyme and trichloracetic acid, and can be restored by replating in magnesium ribonucleate–observations which afford presumptive evidence that these objects do contain a certain amount of PNA. The mitochondrial rodlets which, judging from biochemical analyses have a comparable amount of cytoplasmic nucleic acid, do not however stain by the Gram procedure so that one is forced again to the assumption that either the nucleic acid of the droplet is more "reactive" than that of the mitochondrial substance, which is "bound" or "masked" as a constituent of the original rodlet or that other properties, either chemical or physical, are responsible for the Gram positivity of the droplet. This reaction, though distinctively characteristic for the droplet, tells little therefore of its histochemical constitution.

Though it may seem presumptuous, after a recital of so many uncertainties in the interpretation of the purely qualitative aspect of the constituents of the droplets, to introduce the question of possible quantitative variations which may be observed in the evolution of these structures, it is nevertheless quite certain that the intensity of all of the reactions described varies considerably during the evolution of the droplet. Egg white droplets are, for example, positive to the Baker phospholipid test before they are Gram-positive; both reactions increase in intensity gradually yet irregularly up to the time of their maximum formation when all the droplets are equally and heavily stained. When they disappear, either as a result of *in vitro* autolysis or by *in vivo* metabolic disposal, irregularity in the intensity of stain again appears with the result that in a single cell heavily stained and barely tinged examples are visible at the same time. After the extractions done with ribonuclease at the stage of maximum droplet formation and staining intensity, some of these bodies will be barely visible while a considerable color will persist in others. All these variations in reaction are indeed to be expected in a population of changing droplets having constituents that vary in amount with the metabolic status of the individual complex.

Our discussion so far has been concerned with the cytoplasmic constituents

of the droplets; a short statement may be added as to that other element, the absorbed protein. In the case of one protein, hemoglobin, mere observation of the color of the droplets suffices to indicate its presence. The high carbohydrate content of the ovomucoid of the egg white protein droplets also is clearly demonstrable by the PAS method as contrasted with the weak reaction of droplets forming after the injection of crystallized ovalbumin and blood-derived proteins which contain little glycoprotein. In the case of egg white and the blood serum protein droplets the demonstration of certain animo acids and protein-bound NH_2 and SH groups in higher concentration in the droplets than in the general cytoplasm is also presumptive evidence of the presence of the absorbed protein.

It follows that most methods, the iron-hematoxylin, Gram, and the Baker hematin stain for example, that have been used for the demonstration of the droplets in this and previous studies are no indication of the presence of absorbed protein. The finding of droplets by these methods in late periods of their disappearance does not mean that the protein has not been digested or disposed of, but only that the cytoplasmic elements responsible for that disposal still persist.

It is clear that other, more rigorous, methods must be used to examine into the complexities disclosed by our histochemical techniques. In our first study of droplet formation (6) (Fig. 26), it was observed, in supravital preparations of living tissues, that droplets, freed by mechanical pressure from the cells, floated as persisting objects in the surrounding Locke's or NaCl solution, an indication that they cannot be simple accumulations of the absorbed protein, and this is now amplified by proof that they contain a considerable amount of cytoplasmic phospholipid. This observation of droplet persistence in the aqueous circumambient medium suggests that the techniques of isolation of cell particulates can be applied to an examination of their constitution using immunological, biochemical, and enzymatic methods. Succeeding studies of this series will be concerned with such procedures. One such has already appeared in another place (15).

SUMMARY

Histochemical methods show not only the presence of absorbed protein in the cellular droplet, but also the general identity of its constituent substances with those of the mitochondrial rodlets. Both contain considerable amounts of phospholipid and probably some PNA. The reactivity of the cytoplasmic constituents in droplet and in rodlet are different, however, since in the droplet these substances stain more intensely. As judged by variations observed in this intensity during the evolution of the droplet, these constituents and the absorbed protein vary in their proportions and amount. The droplet would appear to be a highly active center of protein metabolism.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Cells of proximal convolution of a rat, 18 hours after intraperitoneal injection of egg white; fixed in Carnoy's fluid and stained with iron-hematoxylin. No droplets or mitochondrial rods are seen. A preparation of the same kidney is shown in the preceding communication (Fig. 7 of Paper I (1)) after Helly's fixation and Gram stain its cells filled with droplets. × 960.

FIG. 2. Detail of epithelial wall of proximal convolution from the kidney of a rat which had received an intraperitoneal injection of egg white proteins 18 hours previously. Tissue fixed by Altman-Gersh freeze-dry procedure and stained with iron-hematoxylin. The luminal side is uppermost; note the diffusely stained material which fills the lumen. Among the deeply staining rods are scattered large pale staining droplets.

The appearance may be compared to the aspect of these structures when fixed by Helly in Figs. 13, 20 of Paper I (1)). × 4300.

FIG. 3. Egg white drops 18 hours after intraperitoneal injection; stained with Gram after calcium formalin fixation. × 960.

FIG. 4. Duplicate section from the same block of tissue, extracted in pyridine for 24 hours at 60°C. and then stained with Gram. There is no lessening of their Gram positivity. × 960.

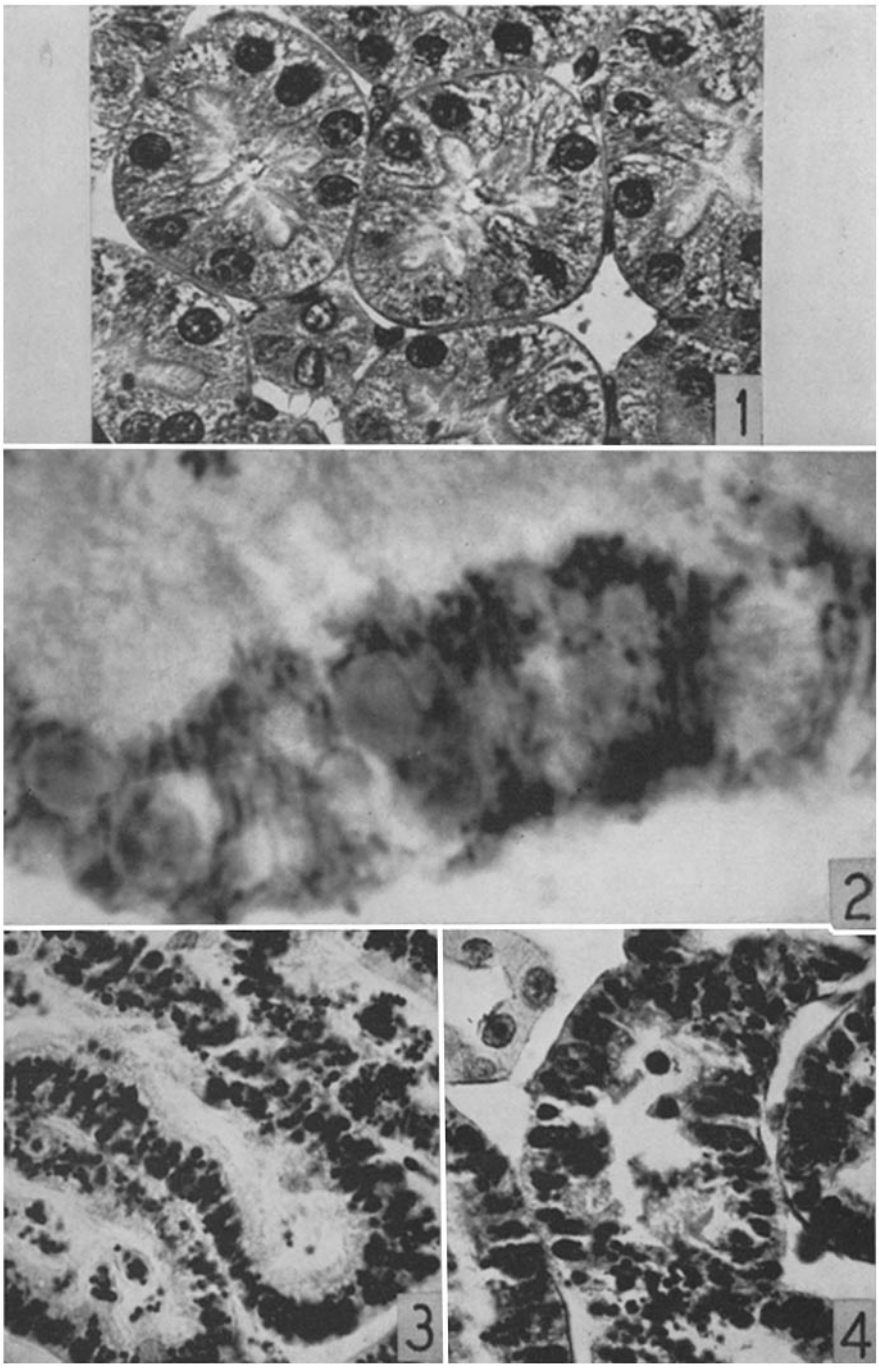

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PLATE 31

FIG. 5. Droplets in a proximal convolution 18 hours after intraperitoneal injection of egg white proteins. Tissue fixed in Giemsa solution and stained with pyronin–methyl green mixture. The droplets stain a deep rose-pink, the reaction of PNA, while the nuclei give the blue-green reaction of DNA. × 960.

FIG. 6. A similar preparation after 2 daily injections of horse serum, showing the same reactions in droplets and nuclei. As is usual, the droplets are much smaller. × 960.

FIG. 7. A similar preparation after a standard injection of bovine albumin. As previously, pink droplets and blue-green nuclei. × 960.

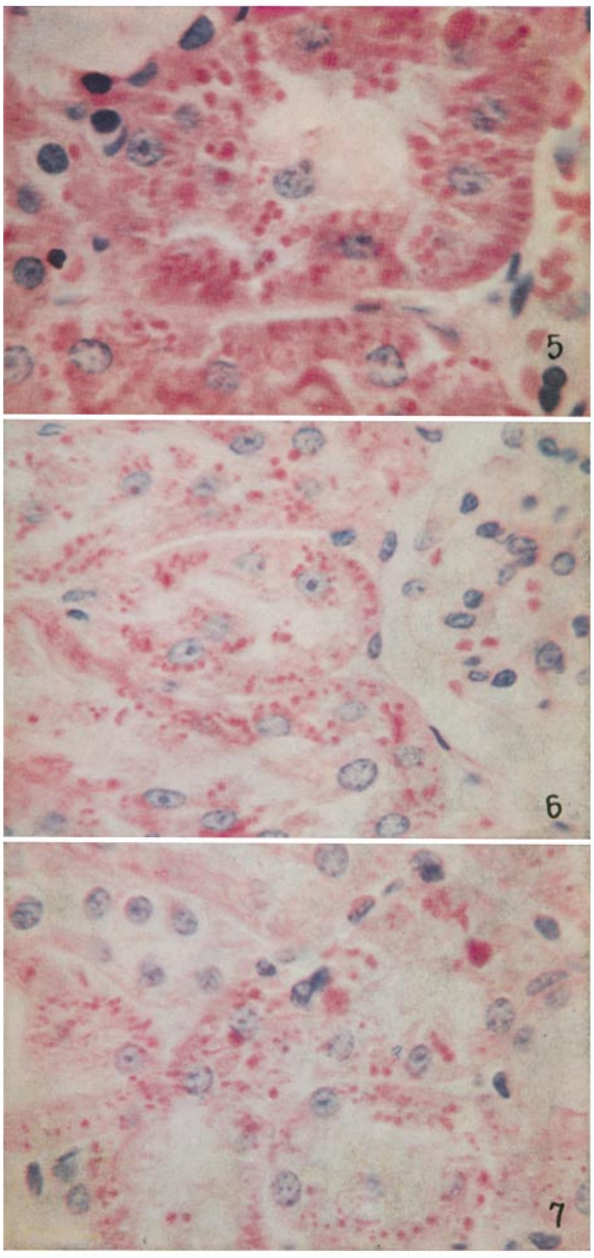

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PLATE 32

FIG. 8. Two cross-sections of a proximal convolution fixed in formalin from the kidney of a normal uninjected rat fixed in formalin and the unstained section photographed at 2537 A. There is a definite band of absorption in the lower two-thirds of the epithelial cells and in certain places a vague striation is evident. The lighter internal band is not the brush border, a structure poorly preserved by formalin fixation, but the luminal apices of the cells into which the mitochondrial rods do not penetrate. There is therefore evidence of some absorption by the rods. The DNA of the nuclei absorbs strongly. × 1000.

FIG. 9. The same section, after extraction with crystalline ribonuclease. The basal zone of slight absorption is no longer evident. The DNA of the nuclei is unaffected. × 1000.

FIG. 10. The same section shown after its final treatment with hot trichloracetic acid. All nucleic acids being removed, the nuclei have disappeared. × 1000.

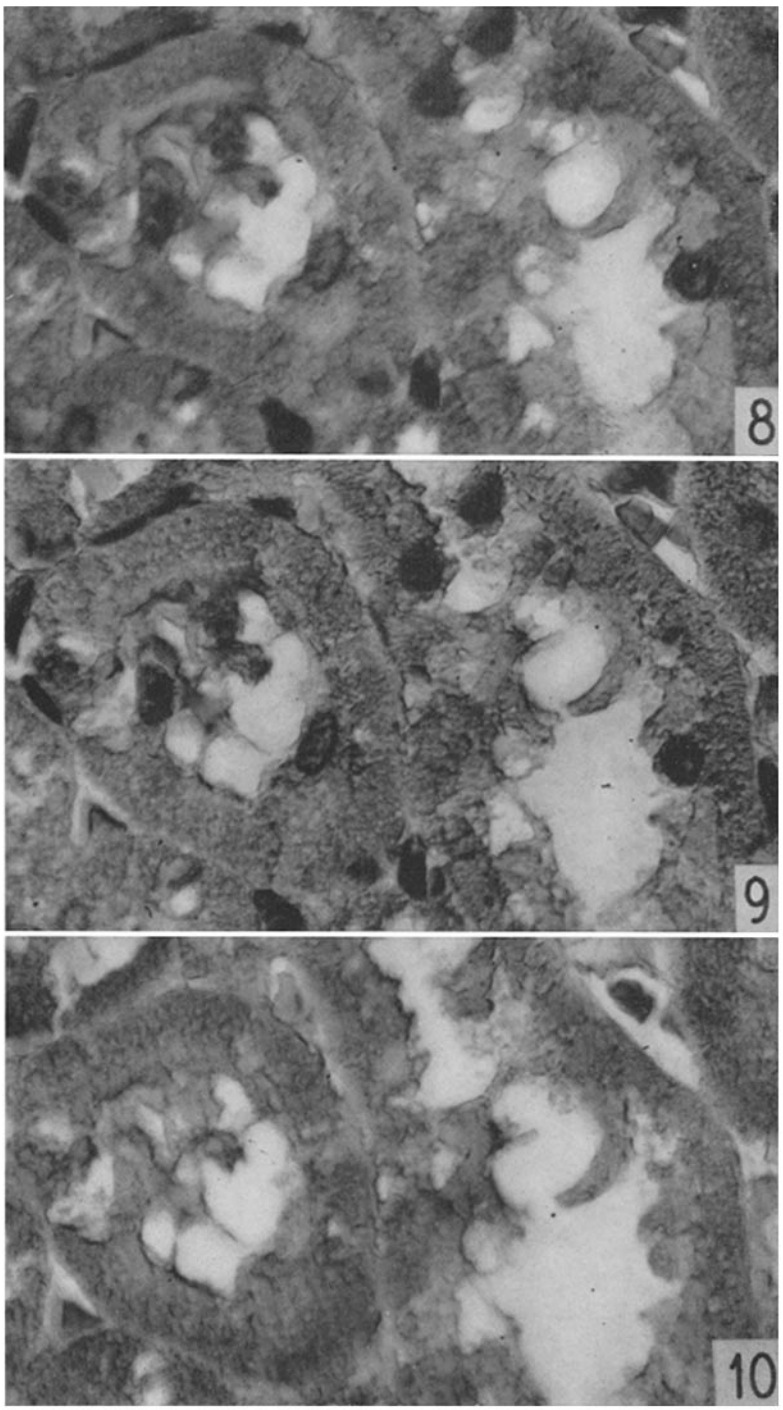

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PLATE 33

FIG. 11. Unstained sections through a proximal convolution of the kidney of a rat 18 hours after injection of egg white, fixed in formalin and photographed at 2537 A. The epithelial cells are distended with droplets which appear as ring forms which lie intermixed with irregular areas of cytoplasmic absorption. (*cf.* Fig. 9 of Paper I (1)). The nuclei absorb strongly. × 1000.

FIG. 12. The same section after extraction with crystalline ribonuclease. No great change is evident in the absorption pattern. × 1000.

FIG. 13. The same section after its final extraction with hot trichloracetic acid. The nuclei have disappeared but the blotches of cytoplasmic absorption and the droplets persist. × 1000.

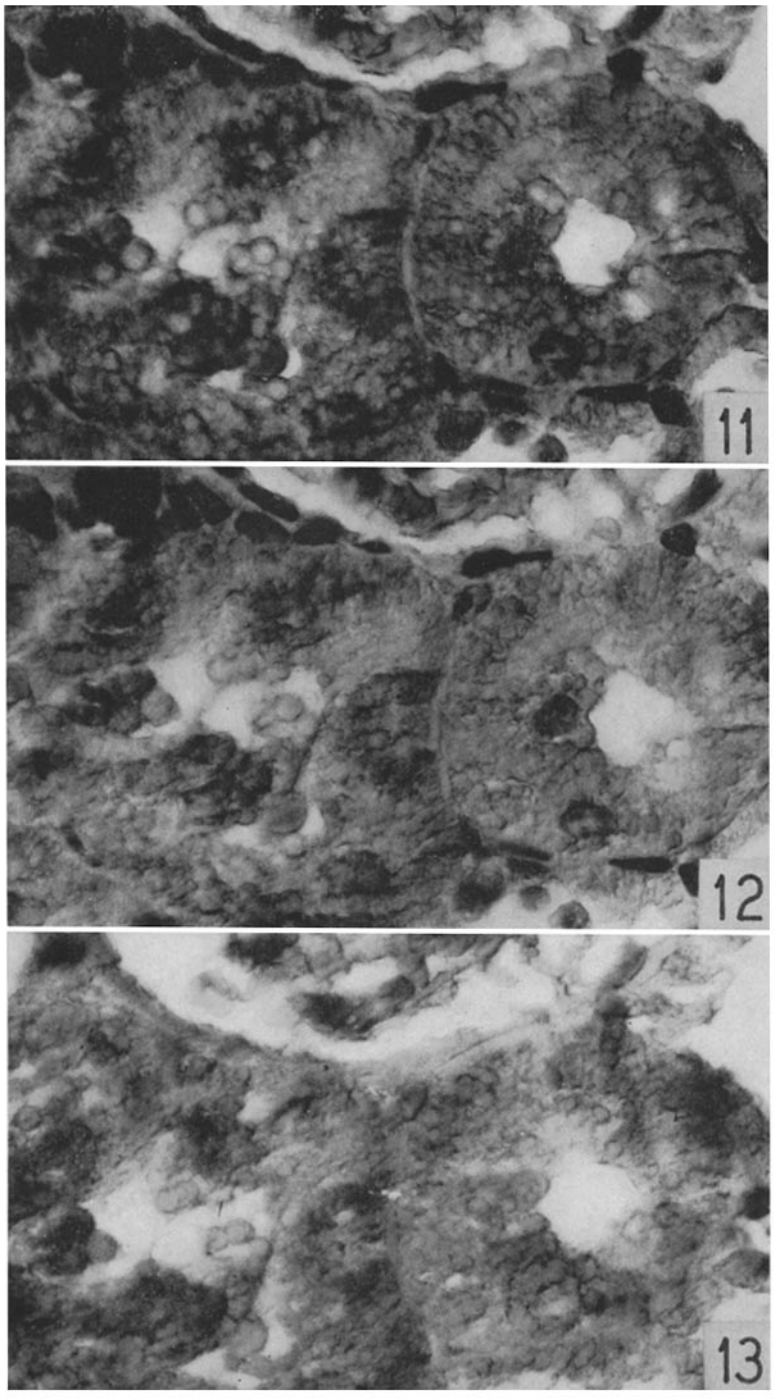

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PLATE 34

FIG. 14. Gelatin frozen section showing droplets in proximal convolution 18 hours after the intraperitoneal injection of egg white; fixed and stained by the Baker acid–hematin method for phospholipids. Above and to the right, the section passes through the convolution in a stretch of tubule free of droplets. The positive reaction of the mitochondrial rodlets is evident. Below, the tubule cells contain equally positive droplets. The appearance of free droplets in the lumen is in part artifact due to inadequate fixation of the protruding apices of swollen cells. × 1000.

FIG. 15. Similar preparation of proximal convolution after the intraperitoneal injection of horse serum. Above, a tubule containing few droplets and many of the original mitochondrial rods. Below, varying proportions of droplets and rodlets. × 1000.

FIG. 16. A similar preparation after injection of bovine albumin. × 1000.

FIG. 17. Tissue from the same kidney as Fig. 14, containing egg white droplets fixed in weak Bouin's fluid and extracted in pyridine at 60°C. and then stained for phospholipid. Neither mitochondrial rodlets or droplets remain. × 1000.

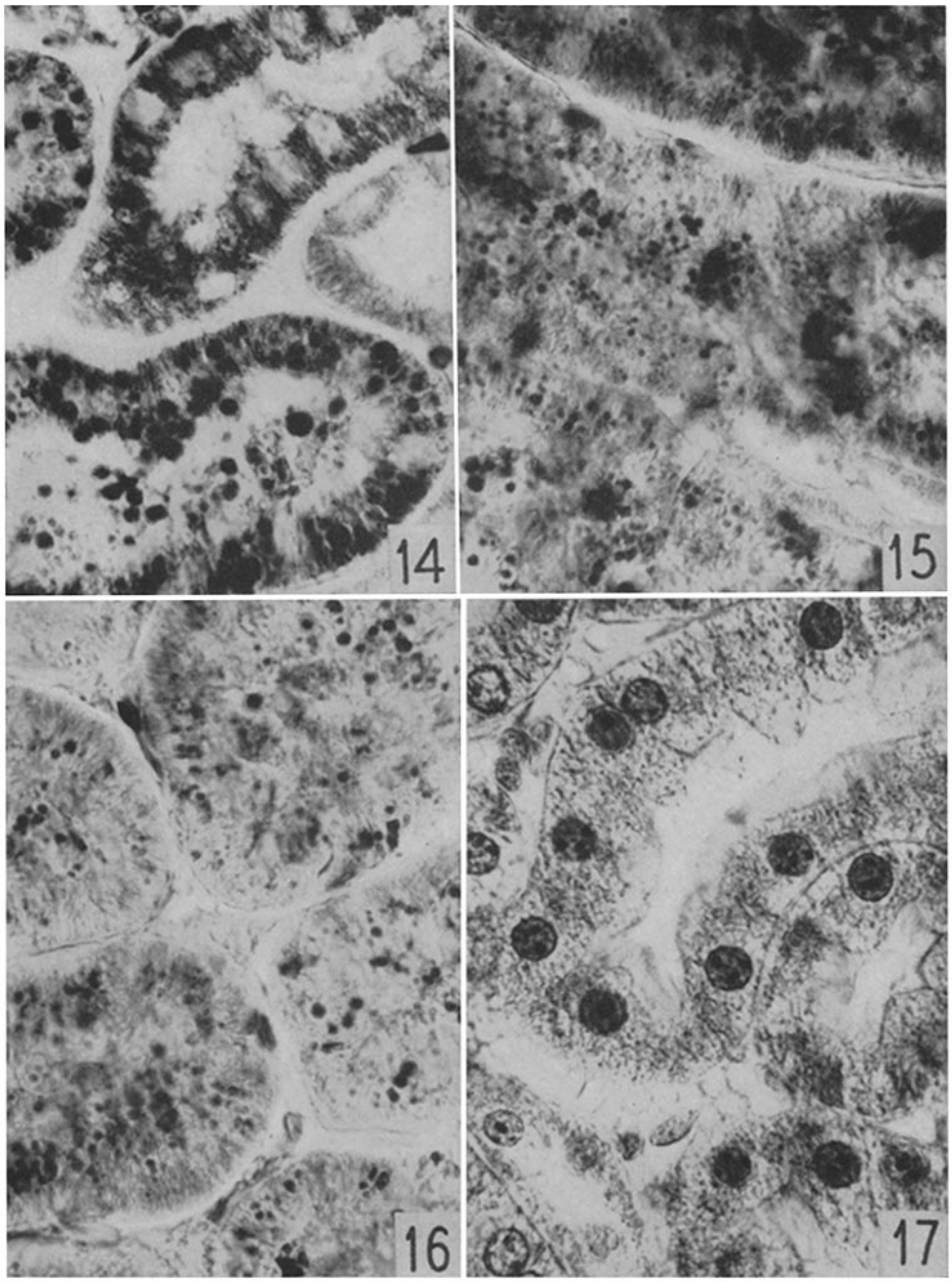

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PLATE 35

FIG. 18. Droplets 18 hours after intraperitoneal injection of egg white proteins, fixed in formalin and stained with the periodic acid–Schiff reaction. The cells are distended with very large droplets which react as strongly as do the brush border, in part disrupted by the swelling of the cells, and the basement membrane. In the original section they were red. × 1000.

FIG. 19. Proximal convolution 18 hours after injection of crystallized ovalbumin; similar treatment. A few pale discrete droplets and irregular flecks of material can be seen in the epithelial cells. They had stained pink. The same tissue, filled with strongly Gram-positive droplets, is illustrated in Fig. 18 in Paper I (1). × 1000.

FIG. 20. Proximal convolution after injection of bovine serum albumin; similar treatment. Although sections of the same kidney, stained by other methods, showed many droplets (Figs. 9, 10 in Paper I (1) only an occasional irregular fleck of stain can be seen in the epithelial cells. The intensity of the reaction should be compared with the strongly positive staining of the brush border. × 1000.

FIG. 21. Proximal convolution 18 hours after 3 daily injections of rat serum; similar treatment. A few definite droplets can be seen in the epithelial cells and more irregular flecks of stain. The many droplets in the cells of this same tissue can be seen in Figs. 11 and 12 in Paper I (1). × 1000.

FIG. 22. Disintegrated but still recognizable droplets following injection of egg white proteins is observed after 48 hours autolysis at room temperature. Only formless debris was seen on staining by the Gram method. (Fig. 43 in Paper I (1)). × 1000.

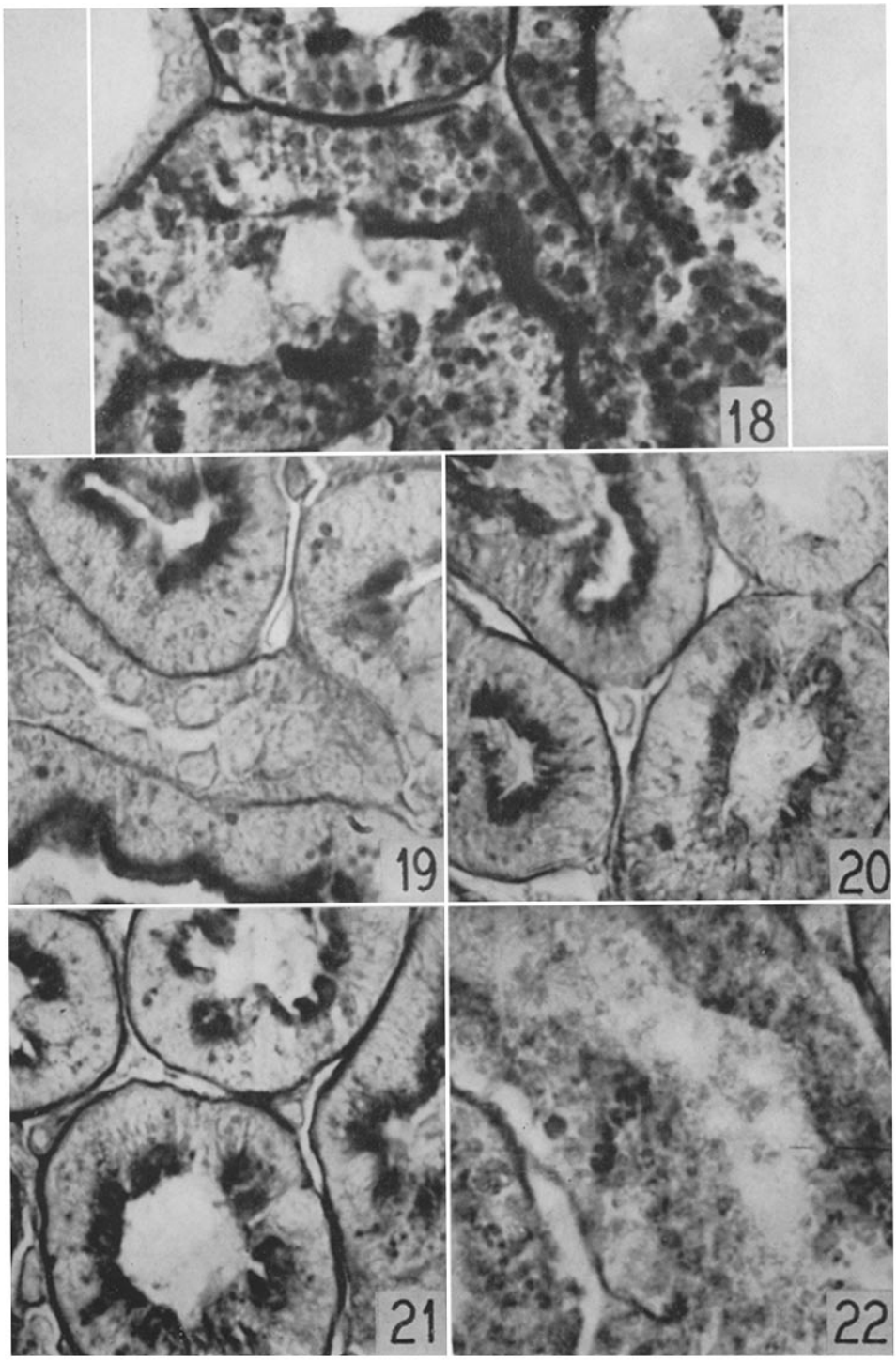

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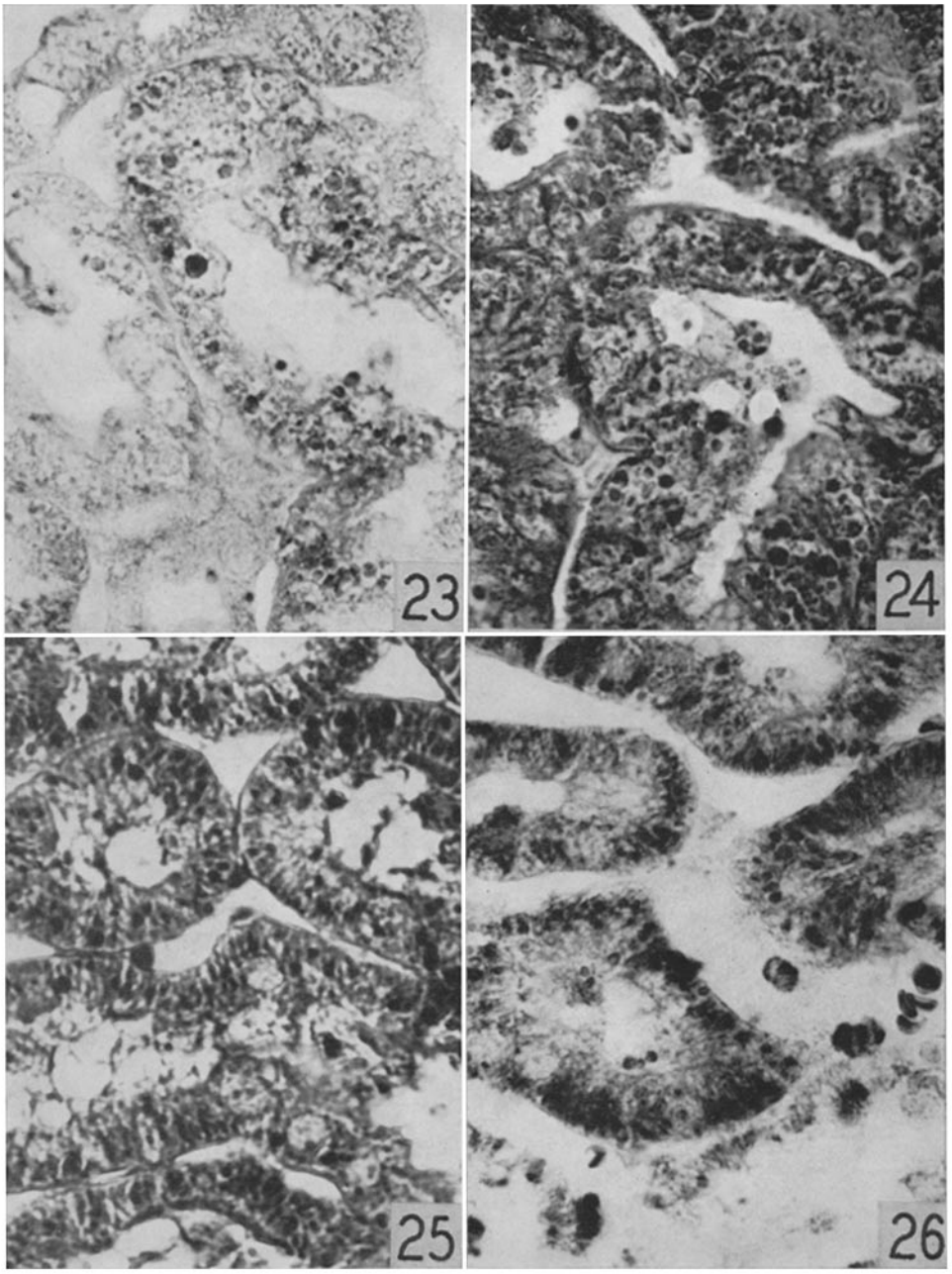
PLATE 36

FIG. 23. Egg white droplets in a convoluted tubule, 18 hours after intraperitoneal injection: Giemsa fixation and staining by Weiss, Psou, and Seligman's method for protein bound NH_2 groups. On the light background of the cytoplasm of the cells, the droplets stand out in dark contrast. × 960.

FIG. 24. The same tissue stained by Danielli's method for tyrosine, tryptophane, and histidine. The droplets were a deep orange-red, appearing on a light orange-yellow cytoplasmic background. × 960.

FIG. 25. Similar preparation of droplets 18 hours after intraperitoneal injection of bovine albumin. The sparser, smaller droplets were deep orange-red. × 960.

FIG. 26. Egg white droplets from the same tissue as Fig. 23, stained with Barnett's method for SH groups. The mitochondrial rodlets were stained lavender-blue and obscured in part the more deeply stained droplets interspersed among them. The general cytoplasm of the cells, where free of rods and droplets, was a light lavender-pink. × 960.



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