CELLULAR MECHANISMS OF PROTEIN METABOLISM IN THE NEPHRON*

I. The Structural Aspects of Proteinuria; Tubular Absorption, Droplet Formation, and the Disposal of Proteins

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Through the work of many investigators, notably Gerard and Cordier (1), Randerath (2), Terbrüggen (3), Smetana and Johnson (4), and Rather (5) much is known of what occurs in the nephron when protein filtering through the glomerular membrane passes down the tubule to appear in the urine. Yet when an attempt is made to integrate these data into a comprehensive description of proteinuria considerable difficulty is encountered (6).

One example of this confused situation may be cited: during certain experimentally induced proteinurias, "droplets," assumed to consist of the absorbed protein, appear in the renal cells which have the general appearance of the "hyaline droplets" that occasionally are found in the human kidney. Yet most human and many experimental proteinurias show no such formations. Are then the droplets in the two instances similar in nature and what relation do they bear to the occurrence of protein in the tubule lumen and to its absorption and ultimate disposal by the renal cells? This and many other questions may be asked; so it would seem that Rather's conclusion (6) is well justified that until more data are available the "process and method of disposal of the protein in the kidney will continue to elude us."

In a preliminary general statement (7) the senior author has shown that in the case of rats excreting egg white proteins, droplets form in a specific portion of the proximal convolution and that they do not react to supravital staining as do the original proteins, but as mitochondrial substances. The latter are apparently derived from the dissolution of the rodlets of the renal cells, an alteration which occurs concomitantly with the formation of droplets. The droplets are ultimately disposed of by the renal cells, decreasing in size and number with the passage of time, though some persist for an indefinite period.

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The interpretation of these observations which serves as the basic hypothesis for the present extension of the investigation just mentioned is that the droplet, presumably a combination of mitochondrial materials[1] and absorbed protein, is the structural aspect of a mechanism which brings together enzyme and substrate for a resulting metabolic modification of the absorbed material. The series of studies to be presented examines this hypothesis and will include a consideration of the morphological, biochemical, and enzymatic properties of the droplets and the other particulate bodies, in particular the mitochrondria, of the renal cell. In the present study we shall consider the morphological features of droplet formation after the administration of a series of proteins, noting differences in their formation and evolution.

General Methods

In their appropriate place the detail of technical methods will be given, but a preliminary statement may be made of certain basic procedures that have been used throughout the investigations.

Since there is considerable variation in the permeability of the glomerular membrane in different species (8) and it is convenient in a study of protein excretion to use an animal in which this process is at a maximum, the albino rat, which normally may excrete up to 1.0 mg. of protein per hour, was used.

The protein solutions were given by intravenous and intraperitoneal injection. By the first procedure the kidney is suddenly flooded with a high concentration of protein which is eliminated in a relatively short time. For example, the intravenous injection into a 150 mg. rat of 1.6 cc. of 7 per cent physiological saline solution of human hemoglobin results in its appearance in the urine in 2 minutes; the excretion is at a maximum in 1 hour and is practically completed in 3 hours. Injection of the protein into the peritoneal cavity on the other hand, results in a delayed appearance in the urine and a relatively flat curve of excretion reaching a maximum in 10 to 17 hours and extending over a period of 2 to 3 days. The cells of the renal tubule are thus exposed to a fairly constant concentration of the material over a considerable time. The differences between conditions following the two methods of administration are essential in certain experiments and failure to consider them has resulted in apparent inconsistency of results previously reported.

Absorption of protein from the peritoneal cavity was usually complete, though occasionally some remained after 18 hours; such animals were discarded. That no essential change occurs in the protein in its passage from the peritoneal cavity into the urine has been shown by Lippman, who reports that after intraperitoneal injection of egg white 90 per cent of the urinary protein was immunologically identical with that injected (9). Our standard experimental procedure was therefore to inject intraperitoneally a set of six rats of 150 to 200 gm. body weight with 16 cc. of a 6 per cent solution and to kill the animal after 18 hours.

The following representative proteins were used: Native hen egg white proteins, crystallized ovalbumin, rat serum, horse serum, bovine serum albumin, human hemoglobin and a

[1] Since the mechanism of staining with Janus green is unknown it may be that physical state, rather than chemical constitution, is the cause of its specificity. The reaction of the droplets therefore is not evidence of the presence of the specific chemical constituents of the mitochondria. Such evidence will be given in the following and other reports of this investigation.

preparation of Bence-Jones protein.[2] It will be noted that the series contains proteins with a variety of physical and physiological characteristics. The first is a mixture that includes the distinctive protein, ovomucoid; the second is a purified member of this mixture. The others are of blood derivation, the first two mixtures and the last three individual blood proteins. All of these substances appeared in the urine after intraperitoneal injection.

The reaction of the animal to the intraperitoneal injection of these, in all but one instance, foreign proteins, must be considered, because such substances have acquired a sinister reputation in respect to possible ill effects on the kidney. No change in the behavior of the animals was noted except for an occasional and transient edema of the face and paws after the injection of egg white. That the use of vasoconstrictors prevents its occurrence (10) suggests other than renal factors as its cause. The short period of the experiments, or the time intervals chosen for injections, eliminated demonstrable immunological reactions.

The question whether the handling of these foreign proteins by the kidney produces "renal damage" will be considered from both the structural and functional viewpoint in a later communication.[3]

The Immediate Absorption of Protein in Non-Droplet Form

There is considerable indirect evidence (11) that suggests that proteins may be absorbed without the formation of droplets. The demonstration of such an absorption meets the practical difficulty that there are few histochemical reactions of sufficient sensitivity or selectivity to demonstrate these substances when they are diffusely dispersed in low concentration throughout the cytoplasm of the renal cells. One protein in our series, however, possesses in its prosthetic group a pigment moiety which allows it to be readily recognized, namely hemoglobin; it was therefore used to demonstrate the earliest phase of the absorptive process. It is known (5) that although droplets of this substance do not form in the renal epithelium until 2 to 3 hours after intraperitoneal injection of hemoglobin, after intravenous injection the pigment appears in the urine in 2 minutes. A period of time may therefore be available for an experiment during which absorbed protein is present in the tubule cells in a diffused state.

Sets of 6 rats were injected intravenously each with 2.0 cc. of a solution of human hemoglobin and killed in 15 minutes. Other sets received two similar injections at a 30 minute

[2] Fresh egg white was diluted with an equal volume of 0.9 NaCl and filtered through gauze, crystallized ovalbumin furnished by the kindness of Eli Lilly Company, bovine albumin powder (fraction V) from The Armour Laboratories, human hemoglobin solution prepared by Dr. Pennell, of Sharp and Dohme, Inc., and the Bence-Jones protein prepared by Dr. Thomas Addis from human urine.

[3] It will be obvious from the experiments that follow that the changes described in the renal cells are not those common to the daily, *i.e.* "physiological," life of a rat. In this sense, the processes are "abnormal." However, the alterations we shall describe are reversible, and evidence will be later given that they are accompanied by little or no disturbance of renal function other than the presence of protein in the urine. Although this ominous sign of renal disturbance has lost many of its original terrors, it is a fact that long continued proteinuria may lead to irreversible structural change in the nephrons and impairment of renal function in both the rat and in man.

interval and were killed 15 minutes later. The bladder urine was deep red in the first group and a dark blackish red in the second.

One of the hazards of all histochemical procedures that attempt to localize dispersed substances in tissues is diffusion during the process of fixation: to avoid this, slices of kidney 1 mm. thick were immediately fixed in absolute alcohol, a strong precipitant of hemoglobin. Other pieces were placed in Helly solution. The two methods of fixation are required because the droplets, though well preserved by bichromate mixtures, are not fixed by alcohol and would therefore be missed if only the latter was used. Paraffin sections were stained by the benzidine method of Ralph.

Examination of sections fixed in Helly solution showed that no droplets had formed in the first group which had lived only 15 minutes following the injection: In the group which had received two injections and had been excreting hemoglobin for 45 minutes a few scattered small droplets were present in the convoluted tubules of some of the animals. In both sets of animals, and particularly in the second, there were deeply colored homogeneous collections of concentrated hemoglobin in tubule lumens and a fine diffuse tingeing of the cytoplasm of the renal epithelium of all cortical tubules with the yellowish color of the benzidine-hemoglobin reaction. The lumens of tubules in the medulla contained hemoglobin but their cells were colorless.

A sharp localization of hemoglobin by the precipitating action of the alcohol was observed. Instead of the weak diffuse staining of all cross-sections of the cortical proximal convolutions of the Helly-fixed material, isolated groups of cross-sections were seen scattered on a background of other proximal cross-sections that were colorless. Each group of stained tubules was oriented around a glomerulus, lying above, below or to one side of it and in each group were a few lumens which contained intensely stained precipitated hemoglobin (Fig. 1).

A more detailed examination of the stained cross-sections of proximal convolutions showed varying degrees of the histochemical reaction. The cells of those whose lumens contained precipitated masses of hemoglobin were heavily stained, though some others containing none were equally dark. The reaction was confined in some to the brush border or to the apical portion of the cells; in others it extended throughout the entire cell body (Fig. 2).

The unusual pattern of positively reacting cross-sections of tubules intermixed with negative, is understandable if one considers first, the *in situ* topographical relations of the cortical proximal convolutions (12) and the fact previously shown by microdissection (7) that the first evidence of absorption with droplet formation occurs in the middle third of the convolution and gradually spreads in both directions along the tubule. Unfortunately, this method could not be used to localize diffuse absorption, as the concentrated HCl of the maceration prior to dissection interferes with the staining method. The focal pattern of positive tubules noted in the sections is, however, compatible only with the conclusion that the diffuse absorption begins in the same limited segment of proximal convolution where droplets later appear.

A similar, though not so strikingly positive, experiment was performed with egg white using the Millon reaction to demonstrate the amino acid, tyrosine, in which it is relatively rich. 2.0 cc. of a 6 per cent saline solution was slowly injected intravenously and the animals killed 15 minutes later. The tissues were fixed in 10 per cent formalin and absolute alcohol, frozen sections cut, and stained by the Millon reaction as described by Pollister and Ris (13).

All of the cross-sections of proximal convoluted tubule were faintly pink, but among them were groups oriented about the glomeruli which stood out in contrast by their deeper color (Fig. 3). None of the tubular cells contained droplets.

The Evolution of the Droplet Phase of Protein Absorption

1. The Formation of Droplets by the Renal Cells:

(a) Variations in Rate of Droplet Formation with Different Proteins.—

In order to follow the formation of droplets, sets of animals were given the standard intraperitoneal dose of egg white proteins and the animals killed after 2, 4, 8, 12, 16, and 18 hours. After fixation in Baker's formalin-calcium and Helly's solution, the sections were stained by the Gram method; for, as we have reported (7), the droplets of absorbed proteins are positive and the rodlets negative.

On microscopic examination of the 6 and 8 hour specimens a few cross-sections of proximal convolution could be seen with occasional, poorly staining droplets. These were increased at 10 hours, but still stained with marked irregularity. In the later periods (12 to 14 hours) there was considerable increase in tubule cross-sections containing them and, in each cross-section, of their number until at 18 to 24 hours a maximum was reached, the tubule cells being then distended with large intensely and evenly stained droplets. Even at this time not all cross-sections of proximal convolution were similar; some contained few or no droplets and none were seen in the medullary terminal segments. (Figs. 4 to 7).

In contrast were the results of a similar experiment with freshly prepared rat serum. As a matter of economy of serum, animals were killed at 4, 8, and 18 hours after injection of the standard dose. In none were droplets found in the renal cells. After injection of the standard amount of crystallized ovalbumin the results resembled those of the egg white experiments in the time of appearance of the droplets, but they were less in number per cell and much smaller. A single injection of the standard dose of horse serum and of bovine serum albumin gave some irregularity in result; the kidneys of some animals of the injected set might contain few or no visible droplets at 18 hours whereas the proximal convolutions in others contained them in considerable numbers.

It is evident that there are differences in the occurrence of droplets depending on protein injected and this observation recalls the concept of Randerath (2, 14) as to the significance of droplet formation. Havermann (15), injecting salamanders intraperitoneally with their own serum, did not observe droplets in the kidney tubules, whereas they were present after a foreign protein was given. Randerath's conclusion is that droplets are only formed when unnatural proteins are absorbed

by the renal cells and on this assumption he establishes a category of disease that includes amyloid infiltration and multiple myelomatosis in which "hyaline droplets" are commonly seen in the tubules of the kidney. These diseases are presumed to be related to some general disturbance of protein metabolism and the droplets to be composed not of physiological substances but of "para" or "meta" proteins. This interpretation, which would explain the commonly observed absence of droplets in a simple proteinuria, overlooks the possibility that quantitative rather than qualitative differences may be the determining factor. The formation of the droplet may depend on the capacity of the cell to handle the load of any absorbed protein rather than on the nature of the absorbed material.

The experiments with rat serum were again performed, the standard injection being repeated on the 2nd and 3rd day. 18 hours later the animals were killed. Gram-stained sections after Helly's fixation showed the tubules filled with droplets (Figs. 11 and 12). Similarly, though not surprisingly, repeated injections of the other proteins filled the tubules more completely.

A series of the proteins examined may thus be established, of which the first is the mixture of egg white proteins and the last rat serum. Egg white produces the most and largest droplets after a smaller dosage; rat serum requires a considerable concentration of protein over a long period before droplets form. The other proteins lie between these extremes.

(*b*) *The Cytogenesis of the Droplets as Observed in Fixed Material.*—In our previous report (7) we have described the changes which occur in the cells of the proximal convolution after the administration of egg white. In summary, concomitantly with the appearance of droplets the mitochondrial rodlets disappeared.

The same inverse ratio between forming drops and disappearing rods was observed in the case of all the proteins of our series. Sets of rats were injected intraperitoneally with the standard dosage of protein and the animals killed 18 hours after one injection of egg white and hemoglobin, 12 hours after 3 daily injections of rat serum and 2 daily injections of bovine albumin, horse serum, and Bence-Jones protein. The tissues were immediately fixed in Helly's solution and paraffin sections (3 to 5μ) stained with iron-hematoxylin.

The appearances noted in the experiments with all the proteins were essentially similar. Adjacent cross-sections of a proximal convolution lying about its glomerulus showed a varied pattern of rodlets and droplets; some contained no droplets and the cells were filled with their characteristic rodlets; at times such a cross-section connected with a glomerular space and was clearly the origin of the tubule. Others contained only droplets and no rodlets and still others variously inverse amounts of both cellular components. Such variation is ex-

plained by the spread of droplet formation along the tubule from the middle third of the convolution (7).[4]

The disappearance of the rodlets may be described as a process of dissolution. The original rodlets, examined either in control animals or in that portion of the proximal convolution of an injected animal which does not contain droplets, are sharply outlined, thin, cylindrical rods that run from the base of the cell into its apical region (Fig. 8). In cells in which droplets are forming the rods are shortened and thickened, with swollen club-like contours. Their original intense black-staining reaction is decreased so that they have no definite outline but fade into a cloud of basophilic material that, giving much the appearance of the ergastroplasma of the older terminology, is diffused throughout the cytoplasm of the cell. In this cloud, the droplets, intensely black, are found. The end-result is a cell filled with droplets with only the basal stubs of the rodlets persisting (Figs. 9 to 21).

(*c*) *The Cytogenesis of the Droplets as Observed in Living Cells.*—In our earlier study of egg white droplet formation (7), phase microscopy of isolated living cells revealed the disappearance of the rodlets. The observation was also made that not only these structures stain with dilute Janus green, a reaction which has been generally accepted as a specific test for mitochondria (16, 17), but the droplets as well are deeply colored by the dye.

This observation suggests that the droplet, previously considered to be simply a globule of absorbed protein, includes an admixture of mitochondrial elements, a conclusion (*cf.* footnote 1) far reaching in its implications, for the mitochondria are rich in enzymes and it is enzymatic action that is responsible for the degradation and disposal of protein. We therefore have extended our observations to droplets of varied protein origin.

Sets of animals were injected by the standard procedures with rat serum, horse serum, bovine albumin, human hemoglobin, and ovalbumin and killed at the appropriate time. Tissue from the cortex was immediately teased in 0.9 NaCl, Locke's solution and fresh rat serum. No particular advantage was noted in the use of the various media. A minute bit was placed between coverslip and slide, gently pressed, and examined by phase microscopy.

In a successful preparation the cortical tissue, glomeruli and tubules, is completely disintegrated and only clumps or free floating cells remain. As the latter are now freed of orienting pressures they become spheres. This disarranges completely the rodlet pattern of the mitochondrial elements which now appear as tangled skeins or short randomly arranged threads. The general appearance of the cells and the changes that occur as the preparations stand have been

[4] Under certain conditions droplet formation may spread into the epithelial lining of Bowman's space and thus produce the effect of "glomerular hyaline droplets." This evidence of the inherent absorptive function of the epithelium will be further considered in our study of the pathological effects of proteinuria.

described and illustrated elsewhere (7). In our examinations the photomicrographs were taken within 15 minutes.

A preparation from an injected rat will show a random population of free cells from all parts of all tubules in the cortex. The great majority are from the proximal convolutions which predominate in this region, both from segments of the convolution which do and do not contain droplets. Identification of a particular cell is not difficult, for the droplets are distinctive and cells from a portion of the proximal convolution not containing them can be distinguished by their brush border from those of other parts of the nephron which are not concerned in droplet formation.

The appearance of the droplets in the living cells is essentially similar after the injection of all the proteins. All degrees of accumulation were present from an occasional droplet to clusters which obscured the nucleus and produced what might be called a "mulberry cell." The droplets varied in size but were from 3 to 6 times the diameter of the spherules that result from the disintegration of mitochondria in old preparations. The egg white droplets were the largest, measuring from 2 to 5μ. Those of other protein origin averaged about 3μ in diameter. The droplets after hemoglobin administration were a light golden brown, the others colorless. As in the stained sections of tubular cells *in situ*, an inverse ratio between the number of thread-like mitochondria and droplets was observed in the single cell.

Supravital staining was performed by mincing the tissue in 0.9 NaCl or Locke's solution that contained Janus green in a concentration of $\frac{1}{25,000}$ to $\frac{1}{50,000}$. The tissue was then placed on a slide and observed with the phase microscope (Figs. 22 to 28).

Both mitochondrial filaments and droplets of all the proteins stained an intense green with the dye, the droplets appearing even deeper in color, an effect doubtless due to their bulk. The nuclei were uncolored. In the hemoglobin experiment, the green of the dye could be seen in the smaller droplets but was obscured in the larger by the golden brown tinge of the protein.

As the preparations stand for 10 to 15 minutes a reduction of the green dye to red was observed in random areas where conditions for the reaction are appropriate (17), the change occurring both in the mitochondrial threads and in the green droplets (Fig. 28).

This observation indicates that the mitochondrial elements within the droplet which were responsible for its Janus green positivity, had retained their ability to catalyze the reduction of the absorbed dye.

2. The Disappearance of the Droplets:

It has been observed by many investigators that changes occur in protein droplets within renal cells with the passage of time: an example is the degrada-

tion of absorbed hemoglobin with the liberation of "free" iron that results in renal siderosis. Rather (5), has described in detail the course of the process: in 3 days a considerable reduction occurred in the number of intracellular droplets and in 6 days almost all demonstrable intracellular hemoglobin had disappeared from the renal epithelium. At 13 days the cells contained only the product of its digestion in the form of hemosiderin. Smetana (8) in his study of proteins labelled with R-salt (egg albumin, serum albumin, and serum globulin) concludes that particles of protein-dye compound remain in the living cells of the tubule until these cells are desquamated: in his discussion he points out that the fate of natural "colloid droplets" may be different since they contain no dye which might interfere with the digestion of protein by the cells. Our results lie between these two extremes.

The disappearance of egg white droplets can be most accurately studied since a single injection fills the cells and so affords a clear-cut departure point for observing the effect of time on a relatively homogenous population of droplets.

Sets of rats were injected intraperitoneally with the standard dosage of egg white proteins and the animals killed at 18 hours, the time of maximum droplet production, and 24, 40 hours and 3, 4, 7, 9, 14, 21, 30, 38, and 51 days later. The tissues were fixed in Helly's and Baker's calcium-formalin solutions and sections stained with iron-hematoxylin and the Gram method.

6 hours after the maximum 18 hour period when the cells were filled with large intensely stained droplets, *i.e.* 24 hours after injection, sections stained with iron-hematoxylin showed only a moderate decrease in the number of cross-sections of proximal convolution that contained droplets, though not all the cells of every cross-section contained droplets. The droplets were smaller, averaging about 2μ in diameter. Although the cytoplasm of the cells was now in part empty, there was no evidence of a regeneration of the original rodlets. In the Gram-stained sections the droplets were still Gram-positive but stained irregularly and less strongly (Fig. 29). At 40 hours there was a sharp decrease in the number of droplets and those remaining were small (Fig. 30). At 72 hours the cross-sections of proximal convolutions of most nephrons showed only a scattering of droplets (Fig. 31) though an occasional group of cross-sections showed that in some nephrons the process of disposal was less far advanced.

From this time on there was a gradual reduction in droplets so that at 51 days only scattered cross-sections could be found containing them. There was now observed a definite increase in rodlets; in a cross-section of tubule that contained occasional droplets, short rod-like structures could be seen in the empty areas of cytoplasm, while other cells of the cross-section which, from the stuffed appearance of the 18 hour specimen, must have been previously filled with droplets and empty of rods, contained the mitochondrial apparatus in its original form and amount (Fig. 32).

It was difficult to compare these results with those of similar experiments

using crystalline ovalbumin and the proteins of blood derivation, rat and horse serum and bovine serum albumin, in which repeated doses were needed to fill the cells with droplets. In general, it may be stated that the process of disposal advanced more rapidly. The droplets after rat serum injection were markedly reduced in 8 days (Figs. 35 and 36) and by 22 days the droplets of the other blood-derived protein were fewer and smaller than those present at a similar time after egg white injection (Figs. 33, 34, 37, 38). This appearance may be due in part to the fact that the egg white droplets were larger and more numerous at the beginning of the experiment. Moreover, there was considerable variation among animals of a single set in contrast to the consistent and maximum droplet change, whether formation or disappearance, noted after egg white injection. Such uncertainties might be expected since the phenomena of droplet formation are minimal when the renal cells are handling blood-derived proteins; a larger and repeated load of protein is required to produce droplets, they are never so large or numerous and consequently their disappearance is less obvious.

In summary, protein droplets of all sorts disappear in large part during the 1st week after their formation but some persist, as Smetana has shown, for an indefinite period. The renal cells dispose of blood-derived protein droplets more readily than those of egg white derivation; further evidence in explanation of this finding is desirable.

3. The Disappearance of Droplets from Renal Cells during Autolysis as Observed in Vitro:

It would seem reasonable to suppose that the disappearance of droplets from the renal cells is due to the action of intracellular enzymes and it should therefore be possible to compare the effect of similar activities *in vitro* in the case of droplets of various protein derivation.

Droplets were produced by the standard procedures in sets of rats with the following proteins: one injection of egg white, three injections of rat serum, crystallized ovalbumin, bovine albumin, and horse serum. Other sets of normal uninjected rats served as controls. At the time of maximum droplet formation the animals were killed and the kidneys cut into small bits that included cortex and medulla, avoiding, in so far as possible, bacterial contamination. Pieces were fixed immediately in Helly's solution and in Baker calcium-formalin solution and the others placed in loosely stoppered vials containing a minute thymol crystal and a few drops of 0.9 NaCl solution. One set of vials remained at room temperature and the other was placed in the incubator at 37°C. At periods of from ½ hour to 3 hours and at 24 and 48 hours the vials were filled with fixing solution. Sections were stained with iron-hematoxylin and by the Gram method. In none of the Gram preparations was bacteriological contamination or proliferation noted.

The observations in the control experiments, in which autolytic processes acted on cellular structures in the absence of droplets, are of interest in two regards, for there exists some difficulty in the practical problem of distinguishing

from postmortem autolytic change in autopsy material such histological entities as "cloudy swelling," "parenchymatous degeneration," and even "hyaline droplets." The control experiments show first the rapidity with which alterations occur in the mitochondrial rodlets of the renal cells and secondly that these changes, though pronounced, do not result in the production of objects with the structural or staining characteristics of either "hyaline" or the experimental absorption droplets.

After 15 minutes at room temperature the clean cut pattern of the mitochondria in sections stained with iron-hematoxylin was confused by a coalescence of the somewhat thickened rodlets and a beginning fragmentation of their filamentous structure. After 30 minutes the rods were resolved into rows of small round granules of approximately 1 to 2μ diameter that had lost their linear orientation and formed a layer in the basal portion of the cell. Neither their size nor their distribution is therefore similar to that of absorption droplets (Fig. 39). Moreover, by the Gram method, they were negative.

The course of the autolytic processes in the droplet preparations was quite different as these objects proved much more resistant, but in varying degree. In sections from the egg white experiments stained with iron-hematoxylin the autolytic disintegration of the rodlets in those cross-sections in which there were few droplets proceeded as in the control animals; the droplets however persisted so that after 3 hours at 37°C. they could still be seen as deeply stained objects (Fig. 40). The Gram procedure showed the detail of droplet disintegration more clearly; during the first 3 hours at 37°C. there progressively developed an irregularity in the intensity of their staining reaction: some individual droplets appeared still deeply stained, others were spotty (Fig. 41). As the cells were no longer completely filled, it was apparent that many had disappeared. These changes were increased in 5 hours at 37°C., many droplets being mere shadows; at 24 hours at room temperature (Fig. 42) only scattered cross-sections could be found in which disintegrating droplets persisted, and after 48 hours at room temperature none were present, only occasional speck-like debris remaining in the swollen and generally autolyzed cells (Fig. 43).

The autolysis of preparations following rat serum injection showed the same disintegration of the droplets but the rate of their dissolution was greatly increased. After ½ hour at 37°C. irregularity of staining was evident and after 1 hour only an occasional cross-section could be found which contained a few feebly stained droplets (Figs. 44 and 45). After 3 hours at 37°C. none remained. The complete autolytic disintegration of the droplets, following injection of crystallized ovalbumin, bovine serum albumin, and horse serum, required the same time (Figs. 46 to 52).

It would appear that there is considerable variation in the ability of the renal cells to autolyze *in vitro* droplets of various protein derivation; rat serum droplets are promptly disposed of while egg white droplets disappear more

slowly. Although the mechanisms are doubtless very different, these observations lend support to the conclusion that *in vivo* the cells can dispose most effectively of droplets of blood protein derivation.

DISCUSSION

When the results of our experiments are integrated with the data of previous investigations it becomes possible to present a tentative explanation of what occurs in the proximal convolution during proteinuria. Our discussion will consist of the statement of an hypothesis, calling attention to the observations so far presented which justify it; further supporting evidence will be given in publications that follow.

During the normal, *i.e.* physiological, life of the rat a certain amount of protein appears constantly in the urine (18). This urinary protein is derived from the plasma proteins presumably by passage through the glomerular membrane, since it is impossible to produce proteinuria in animals that have no glomeruli (19). It is not identical in its constituents with the proteins of the plasma, however, as it consists of only the larger bodies, the globulins (11). The albumin has disappeared. If rennin is given, depressing the absorptive activity of the tubular epithelium, and probably increasing the permeability of the glomerular membrane as well (20), the albumin appears and the urinary protein is essentially identical with plasma protein in its constituents (11). A similar situation exists in man; there is normally a constant proteinuria (30.5 to 49.6 mg./hour) and the A/G ratio of the urinary proteins is the reverse of that in the plasma (21).

It follows therefore that albumin must be continuously absorbed from the tubule fluid in the rat and man at some point in the nephron, and that since a continuing accumulation of protein in the renal cells is impossible, it is in some way disposed of, possibly after intracellular modification, and passed on eventually into the blood stream.[5]

The filtered protein, as our experiment with hemoglobin and egg white demonstrated, is absorbed by the cells of the proximal convolution. This activity is first evident in the middle third of the convolution and spreads along the tubule as the absorption increases or is long continued. The absorbed protein is diffusely dispersed in the cytoplasm of the renal cell: it can be seen, on what appears to be its entrance into the cell, confined to or concentrated beneath the

[5] The amount of material handled by these processes is considerable. Assuming that in man the 180,000 cc. of glomerular filtrate in 24 hours contains plasma proteins in a concentration of only 0.010 gm./100 ml. filtrate, far below present methods of measurement, then some 18 gm. of protein must be absorbed. But this represents only a small part of the total load of utilizable N that is handled by the nephrons, since all the amino acids of the plasma pass the glomerular membrane and are absorbed in an amount equivalent to the amino acid content of some 112.0 gm. of protein. It would seem therefore that in a single day about twice as much utilizable N passes through the walls of the nephrons, undergoing meanwhile modifications of which we do not know, as is contained in the average daily total protein intake.

marginal brush border and, in what would seem to be later stages, filling the entire cell body. There is no alteration of the cytological pattern of the absorbing cells; to ordinary methods of observation they appear empty and their mitochondrial rodlet apparatus is unchanged. Recent experiments with rats, using fluorescent antibodies of bovine serum albumin, have also shown the presence of diffusely absorbed protein in "tubule cells" (22), and it well may be that the swollen, faintly granular, eosinophilic convoluted tubules, not infrequently seen by the pathologist as "cloudy swelling" in the kidney of various proteinurias, are evidence of a similar diffuse absorption of plasma proteins.

What has been described so far may be all that is seen in the way of structural and functional correlations in proteinuria; it is a description that applies to the kidney of the normal rat and to many proteinurias that develop for various reasons, including increases of permeability due to glomerular damage, in man. As we shall have need to distinguish these phenomena from those of other conditions of protein absorption, we shall refer to what has been described as the early or diffuse phase of proteinuria.

What happens to the absorbed protein that diffusely fills the renal cells must remain for the present a matter of speculation: we know that one protein, hemoglobin, which is absorbed by the renal cells, disappears as an entity leaving its Fe as an intracellular deposit. We can be reasonably certain that there is a limit to the capacity of the renal cells to accumulate protein and that they must therefore dispose of it by passing it on in some form to the blood stream. In other words, there is of necessity a *metabolic* component in what at first glance appears to be the purely *absorptive* first phase of the proteinuria.

If the capacity of the simple absorptive and metabolic mechanisms of the first phase of the proteinuria, with its minimum structural change, is exceeded a second phase follows in which accessory cytological mechanisms appear that produce cellular alterations, namely the appearance of "protein" or "hyaline" droplets. Concomitantly with the formation of the droplets and proportionately to their increase, the mitochondrial rodlets of the absorbing cells disappear and the region of their dissolution is filled with droplets.

The observations on droplet formation originally made by us in both fixed tissue and by phase microscopy in living cells (7) after injection of egg white proteins have been confirmed by others (23) and they are now extended to droplets of various protein derivation. Our experiments here reported also indicate that the droplets are not composed of the absorbed protein alone; for like the egg white droplets, these structures react by supravital staining with dilute Janus green as do the mitochondrial rods which, disintegrating, have liberated their substance into the general cytoplasmic reservoir of the cell. The reduction of the dye within the droplet to its pink form indicates that catalysts (enzymes) which are known to exist in the original mitochondria have been transported to the droplet.

It would appear therefore that the droplet is an intracellular structure of

considerable complexity, containing the absorbed protein and mitochondrial elements (*cf.* footnote 1) and some of its enzymes; thus the droplet becomes the structural locus of an intracellular metabolic process for the disposal of the absorbed material, acting as a mechanism by which substrate, protein, and enzyme are brought together.

The fundamental intracellular processes that we have described as the early diffuse phase and the late droplet phase of proteinuria occur in the handling of all proteins, whether these are autogenous plasma proteins or grossly foreign ones such as egg white. The relative part played by the two phases depends, however, on the ability of the renal cell to handle the absorbed material. Thus the injection of a moderate amount of egg white proteins resulted, after a brief period of the structurally negative first phase of diffuse absorption, in the production of a maximum of droplets which, although most were gradually destroyed by the enzymatic activity of the renal cells, persisted for weeks. The single injection of a similar amount of the rats' own serum proteins produced no droplets, there being no structurally visible expression of the absorption or disposal of the protein. Three, or in some instances four times as much rat serum had to be given over a period of successive days before the diffuse phase was passed and droplets appeared in the renal cells. The droplets were never so large nor were the cells so filled as in the egg white proteinuria, nor did the droplets persist so long in the cells but were digested more completely and disappeared more rapidly.

The varied appearances of the droplet phase, and even the circumstance of its occurrence depend, therefore, on the metabolic capabilities of the renal cell to dispose of the absorbed proteins. As our experiments with the autolytic destruction of the various protein droplets in tissue slices indicated, the renal cells contain enzymes that digest autogenous serum protein droplets promptly while the destruction of egg white protein droplets is accomplished only after hours of incubation.

In conclusion, droplet formation is an accessory mechanism in the metabolic process by which the renal cell disposes of absorbed protein; quantitative not qualitative factors account for its development, a very little egg white or a large amount of serum protein producing the same type of intracellular reaction.[6] The intracellular phenomena that accompany proteinuria, previously described under the various disparate categorical entities of absorption, cloudy swelling, athrocytosis, hyaline droplet formation, and even as "diseases of the kidney tubule" (acute nephrosis), or as evidence of a general disturbance of the "metaprotein metabolism," become thus an integrated and unified process.

[6] It would seem reasonable to suppose that proteins passing the glomerular filter during spontaneous proteinurias are more "natural" and therefore more easily "handled," than our artificial preparations of homologous rat serum. Hence the threshold of the ensuing droplet formation would be higher, thus accounting for the infrequency with which droplets are seen in human proteinurias.

SUMMARY

When proteins pass the glomerular filter they are in part directly absorbed by the epithelial cells of the proximal convolution of the nephron with no apparent alteration of the cytological pattern. If the capacity of the tubule cells to thus absorb protein from the tubule fluid is exceeded either by the amount or the nature of the protein the accessory mechanism of droplet formation occurs. This accessory mechanism is an intracellular process in which cytoplasmic elements, the mitochondria with their enzymes, and the absorbed protein combine to form droplets. As the droplets form and then disappear from the renal cells their evolution presents a constantly changing picture depending on the varying nature of their protein and cytoplasmic content.

The droplet is therefore not a cytological structure of fixed characteristics (hyaline droplet) but a locus of metabolic activity and varied structural aspect.

As this publication goes to press, Dr. J. Rhodin sends us his thesis from the Department of Anatomy of the Karolinska Institutet, Stockholm, Sweden, in which changes in the mitochondria of the renal cells of the proximal convolution of the mouse are described as seen by electron microscopy. His final conclusion reads: "Electron microscopic analysis on sections has confirmed the assumptions of Oliver (1948) . . . namely, that intraperitoneally injected egg white . . . on reabsorbing, causes changes that may be localized to the mitochondria . . . of the proximal convoluted tubules." Three stages in these changes are described: The first, in which the coalescence of mitochondria and "a finely grainy substance" is seen forming "large granules." The second, in which "the characteristic mitochondrial structure has been lost and the cell is filled up by finely grainy granules, 2–4 μ in size. The number of mitochondria . . . and the area of the mitochondria have been markedly decreased. In the third stage, the size of the granules has decreased." The size and appearance of Dr. Rhodin's "large granules" correspond to our "droplets."

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EXPLANATION OF PLATES

PLATE 17

The early phase of proteinuria with diffuse absorption of protein.

FIG. 1. Cortex of kidney from rat injected intravenously 15 minutes previously with hemoglobin solution, fixed in absolute alcohol, and sections stained with benzidine. The epithelium in the cross-sections through the proximal convolutions of two nephrons has been diffusely stained by the absorbed hemoglobin. There are no droplets present. Photomicrograph at 370 to 490 mμ. × 98.

FIG. 2. Same preparation. Detail of three sections of proximal convolution showing diffuse absorption of hemoglobin with highest concentration at the lumenal border of the cells. Fine granules of hemoglobin precipitated by the alcohol are present in the lumen. × 586.

FIG. 3. Cross-sections of a proximal convolution from a similar experiment with intravenous injection of egg white solution instead of hemoglobin. Tissues fixed in 10 per cent formalin and stained with the Millon reaction. Three cross-sections reacted positively, a deep pink as indicated by the dark gray of the photograph; the others show only a faint cytoplasmic reaction. × 684.

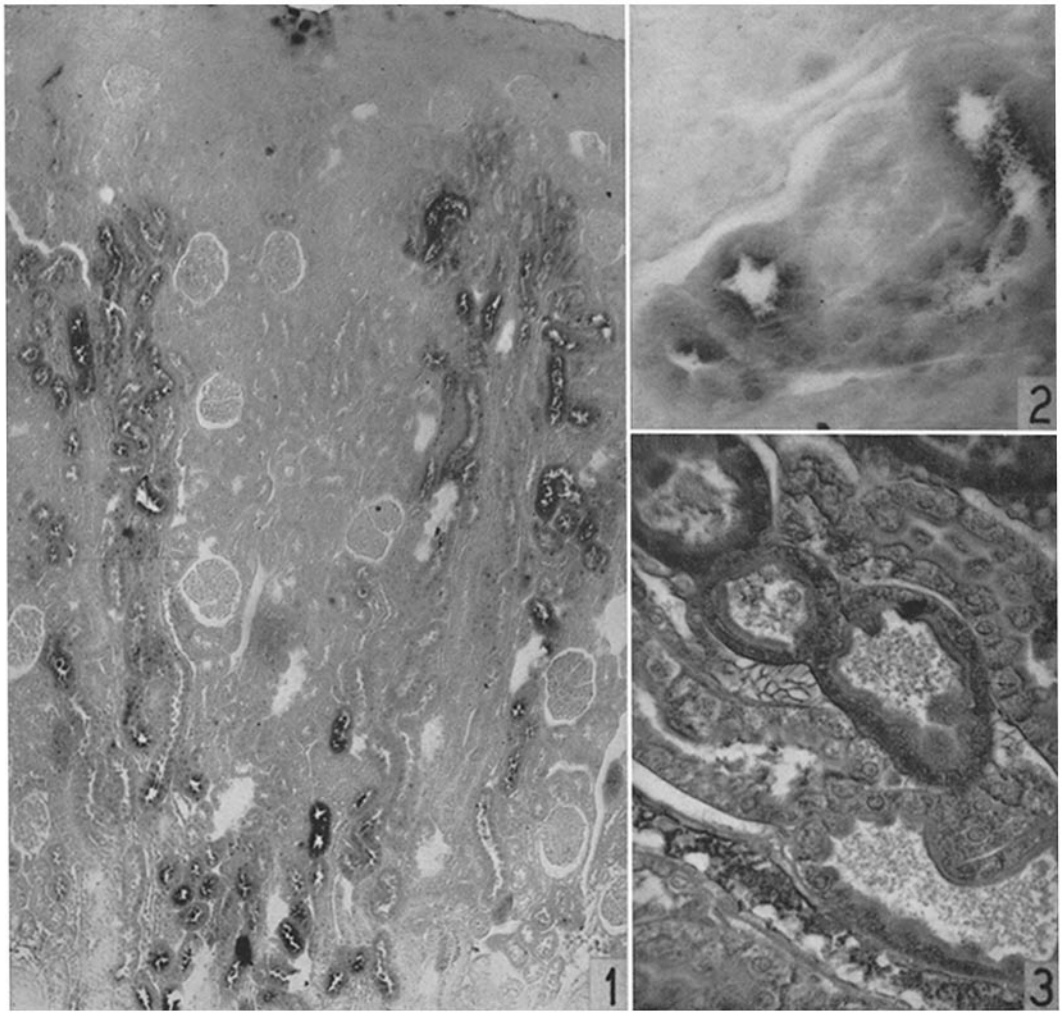

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PLATE 18

The late phase of proteinuria with protein droplet formation.

FIG. 4. Kidney of rat 8 hours after intraperitoneal injection of egg white; tissue fixed in calcium-formol solution, sections stained by Gram method. Above, cross-sections of a proximal convolution contain a few scattered Gram-positive droplets. × 960. Below, detail of epithelium showing minute aggregations of Gram-positive material and their coalescence to form droplets. × 1800.

FIG. 5. A similar preparation from a rat 10 hours after injection. There is a great increase in droplets of various size. The droplets stain irregularly, some heavily, others only faintly × 960.

FIG. 6. A similar preparation from a rat 14 hours after injection. A further increase in droplets is evident. The droplets are larger and stain more deeply and evenly, though many faintly tinged are still present. × 960.

FIG. 7. Similar preparation from a rat 18 hours after injection. The droplets, though of variable size, now all stain deeply and evenly with Gram method. × 960.

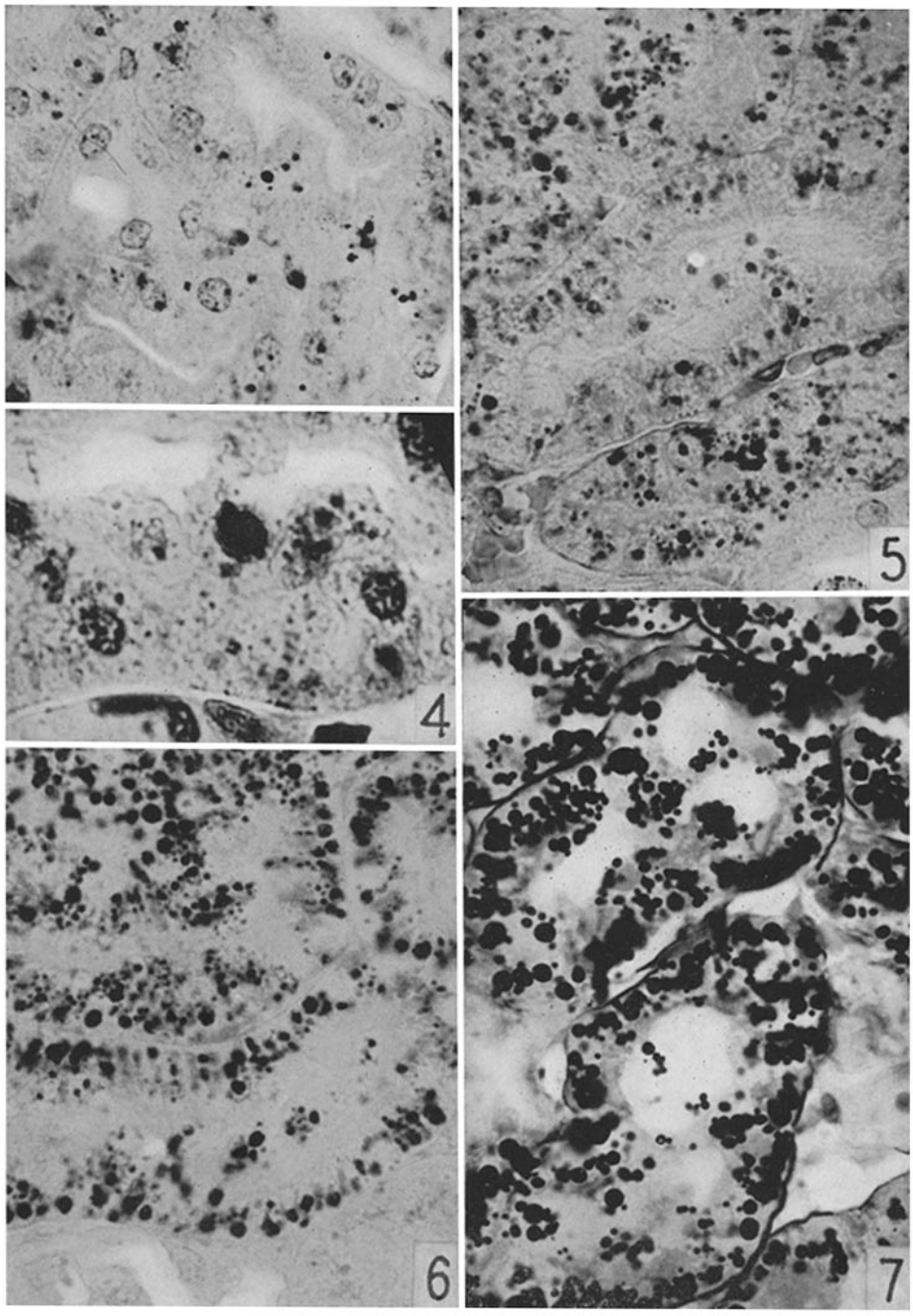

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PLATE 19

The cytogenesis of the protein absorption droplets.

FIG. 8. Rodlets in the cells of the proximal convolution of a normal control rat that had received no protein. Isolated portions of tubule wall, lumen side uppermost. The closely packed cylindrical rods form a dense palisade that fills the lower two-thirds or more of the cell. Helly fixation, iron-hematoxylin stain. × 4300.

FIG. 9. Similar preparation showing a cross-section of a proximal convolution from the kidney of a rat which had received an intraperitoneal injection of bovine albumin 18 hours previously. Among the disintegrating rodlets are seen the heavily stained droplets. × 2000.

FIG. 10. From the same kidney, tissue fixed in formalin and stained with Gram method. Portions of two proximal convolutions filled with droplets that are strongly Gram-positive; the rodlets are negative and do not appear. × 1400.

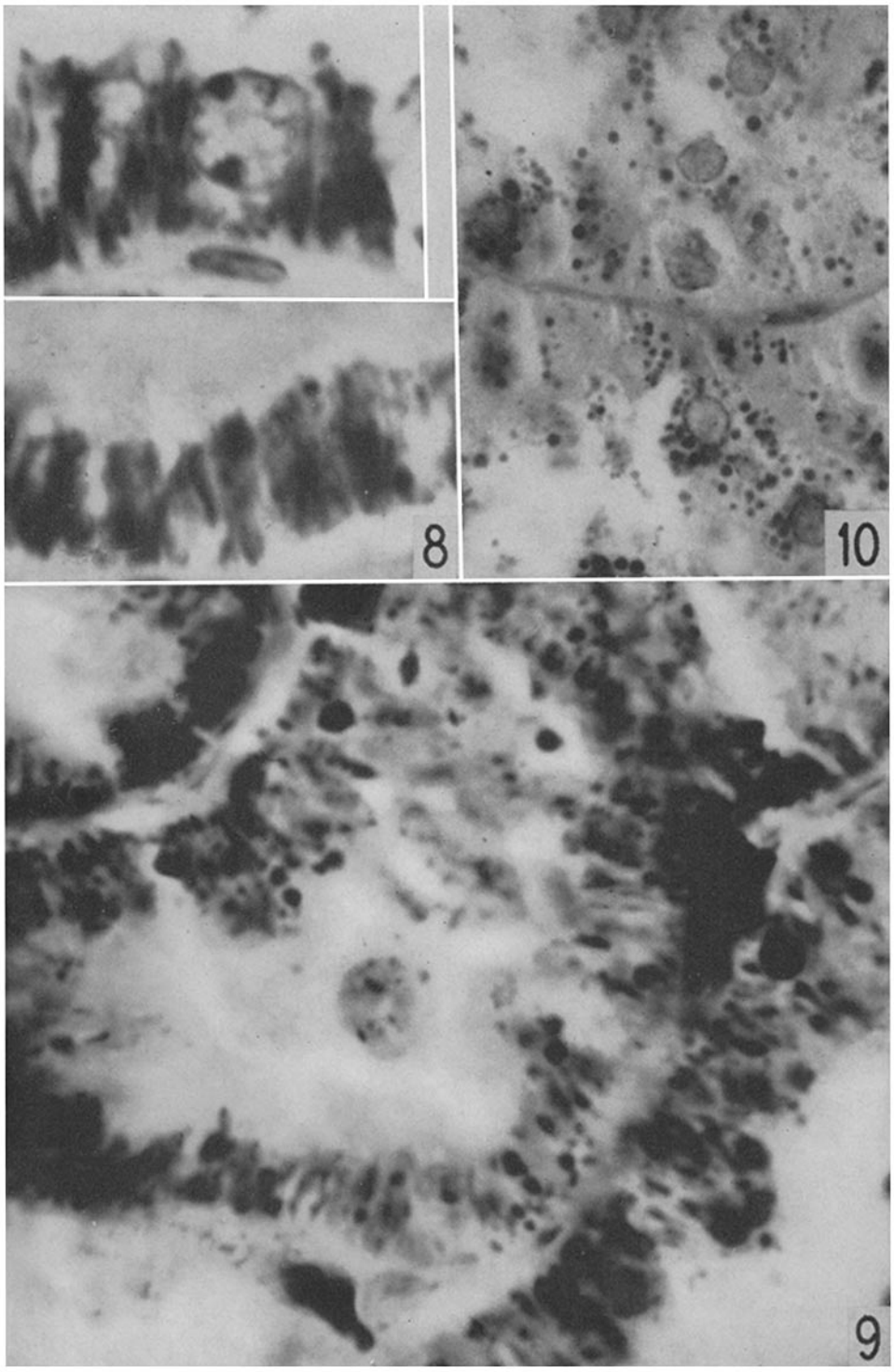

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PLATE 20

FIG. 11. A preparation similar to that of Figs. 8 and 9 showing portion of a proximal convolution from the kidney of a rat which was killed 18 hours after it had received three daily injections of rat serum. The rodlets have completely disappeared, leaving a cloud of basophilic matter in which the deeply stained droplets are scattered. × 4000.

FIG. 12. The same kidney, fixed in formalin and stained with the Gram method showing positive droplets and no rodlets. × 1000.

FIG. 13. Detail of Helly-fixed, iron-hematoxylin-stained proximal convolution from rat which had received an intraperitoneal injection of Bence-Jones protein 18 hours previous to sacrifice. Two isolated portions of the epithelial wall of a proximal convolution, lumen side uppermost. Amidst the dissolution of the rodlets many large deeply staining droplets are seen. × 4300.

FIG. 14. Gram stain of formalin-fixed tissue from the same kidney showing Gram positivity of the droplets. × 1000.

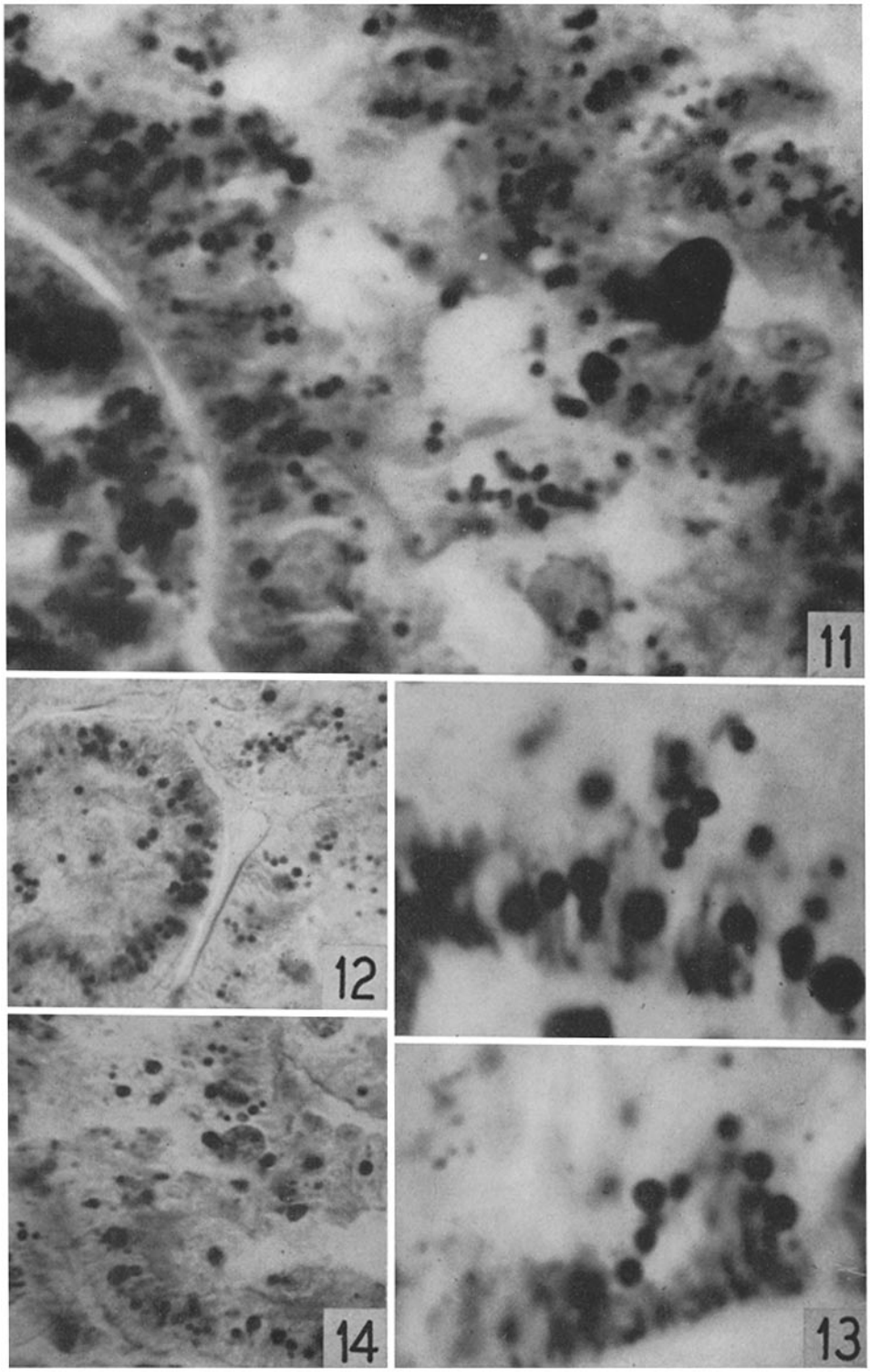

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PLATE 21

FIG. 15. Cross-sections of a proximal convolution from the kidney of a rat killed 18 hours after intraperitoneal injection of horse serum. In the upper right the section passes through the upper portion of the convolution which does not contain droplets; in the others there is almost complete dissolution of the mitochondrial rodlets and many droplets. Helly's fixation, iron-hematoxylin stain. × 1000.

FIG. 16. Section from the same kidney fixed in formalin and stained with Gram method. The Gram-positive droplets alone appear. × 1000.

FIG. 17. Helly's fixation and iron-hematoxylin stain of proximal convolution 18 hours after injection of crystalline ovalbumin. Small droplets, as compared to those forming after egg white (*cf.* reference 7, Fig. 12), fill the tubule cells. × 1000.

FIG. 18. From the same kidney fixed in formalin and stained by Gram method. × 1000.

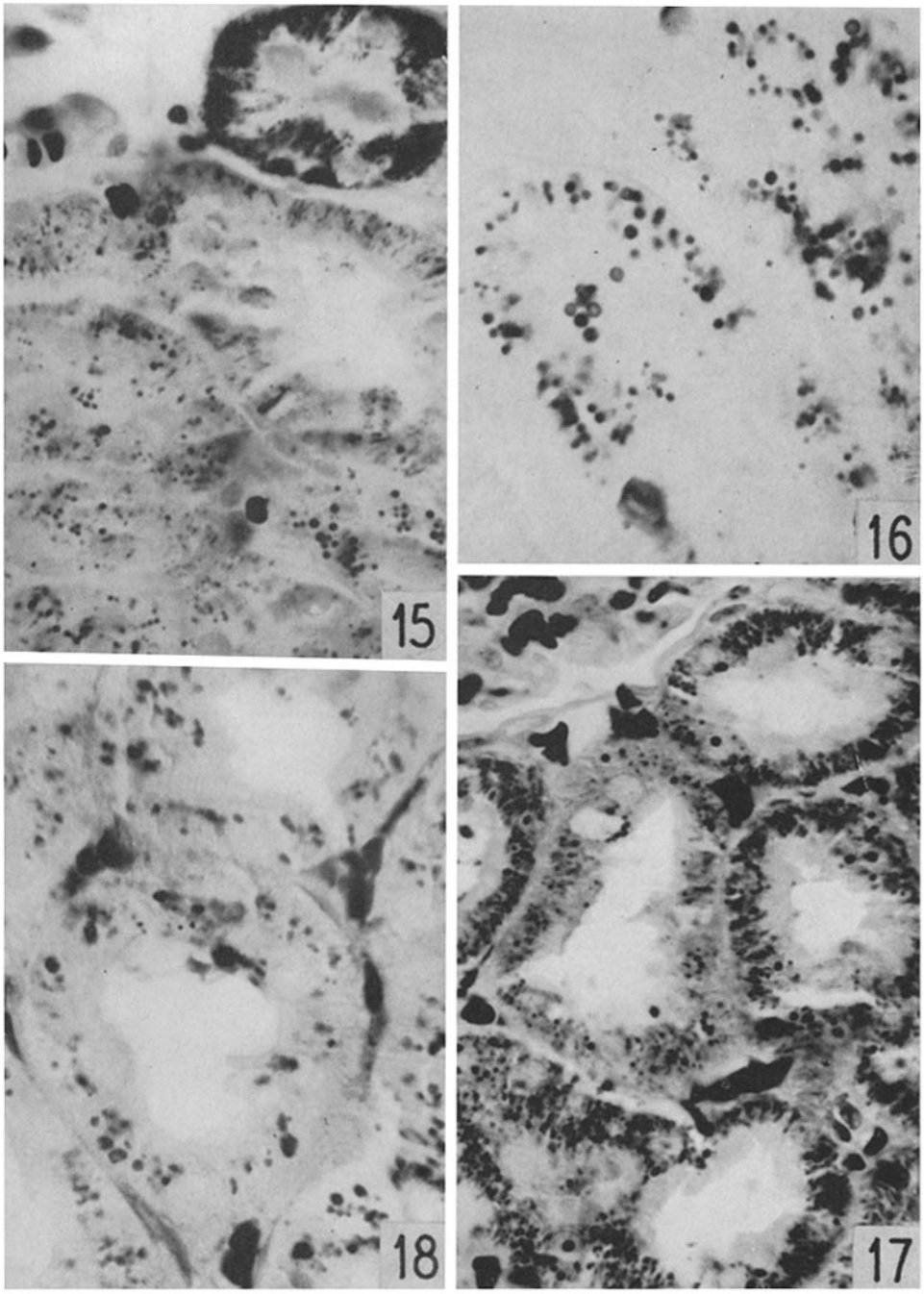

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PLATE 22

FIG. 19. Proximal convolutions showing rodlets and a moderate number of droplets 18 hours after intraperitoneal injection of human hemoglobin. Helly's fixation and iron-hematoxylin stain. × 1400.

FIG. 20. Detail from the same slide showing the early alteration in the dissolution of the rodlets and the formation of droplets. Two isolated segments of the wall of a proximal convolution, lumen side uppermost. × 4300.

FIG. 21. The same kidney fixed in 10 per cent formalin and Gram stain. ×1400.

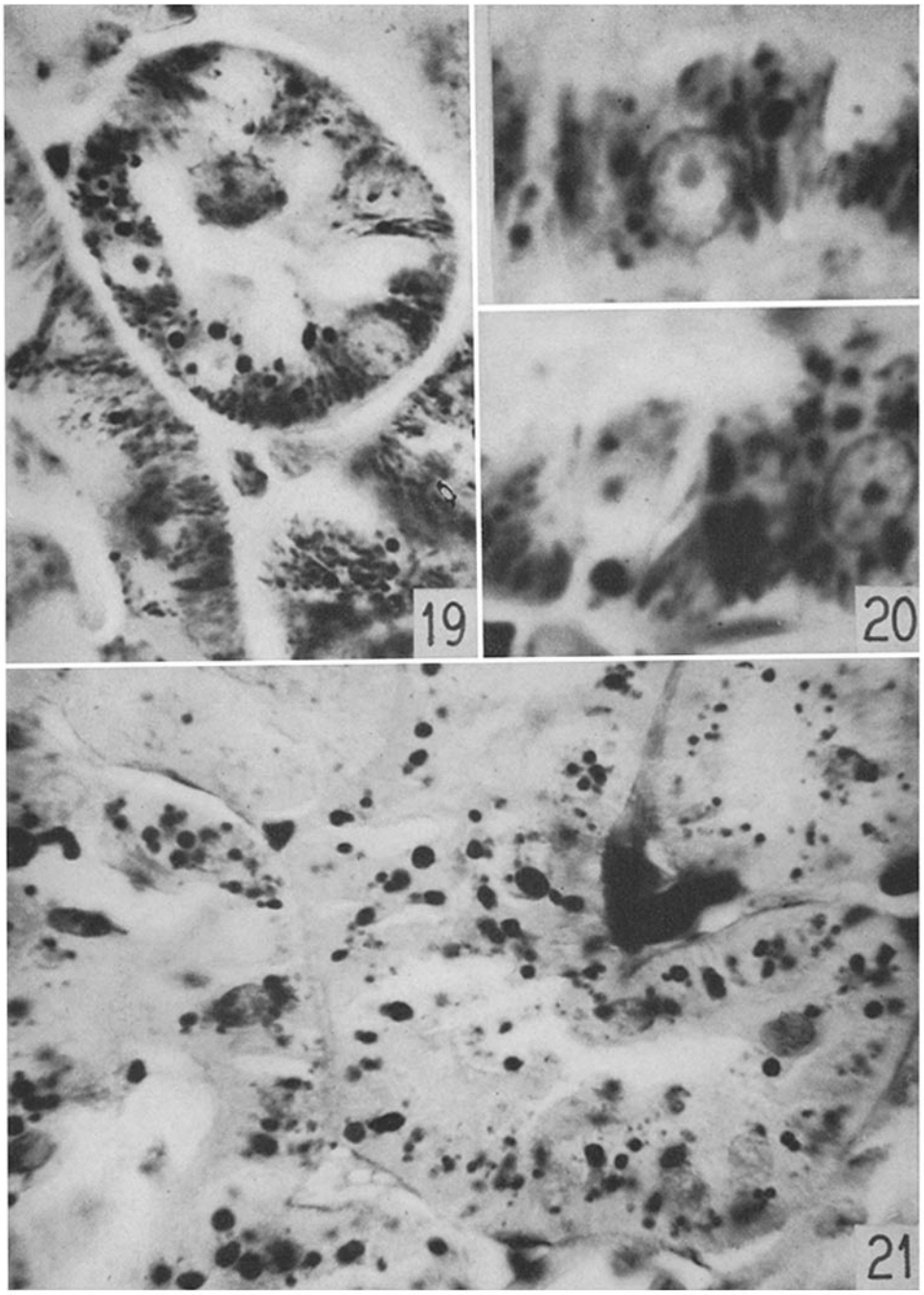

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PLATE 23

Kodachrome microphotographs taken with dark phase illumination of living isolated cells from the cortices of rats injected intraperitoneally with various proteins 18 hours previous to sacrifice and stained supravitally with $\frac{1}{50,000}$ Janus green. In the round, free floating cells the droplets not in the exact focus of the kodachrome cause some diffuse coloration of the cytoplasm; this unavoidable photographic artefact is particularly prominent in Fig. 28 where the natural color of the hemoglobin predominates.

FIG. 22. Cell from proximal convolution of tubule containing no droplets (Fig. 15). The radiating brush border surrounds the cell which is filled with filamentous blue-green staining mitochondria. Since the cell is alive, the nucleus is unstained. × 2500.

FIG. 23. Cell containing droplets 18 hours after the injection of egg white proteins. The mitochondrial filaments have disappeared and the droplets are deeply tinged with Janus green. × 2500.

FIG. 24. Similar preparation showing absence of mitochondria but presence of Janus green–positive droplets after injection of crystallized ovalbumin. × 2500.

FIG. 25. Similar preparation after injection of horse serum. × 2500.

FIG. 26. Similar preparation after injection of bovine albumin. × 2500.

FIG. 27. Droplets following injection of egg white proteins; 20 minutes after contact with Janus green. The droplets are swollen and in each there is a reduction of the dye to its pink form. Note that this reduction is localized to the droplet and is not a reaction generalized throughout the preparation, an indication that some specific catalyst is contained within the droplet. × 2500.

FIG. 28. Droplets after injection of human hemoglobin stained with Janus green. The natural color of the hemoglobin obscures the greenish tinge of the dye, though its presence is evident in some droplets by a muddy greenish brown discoloration. × 2000.

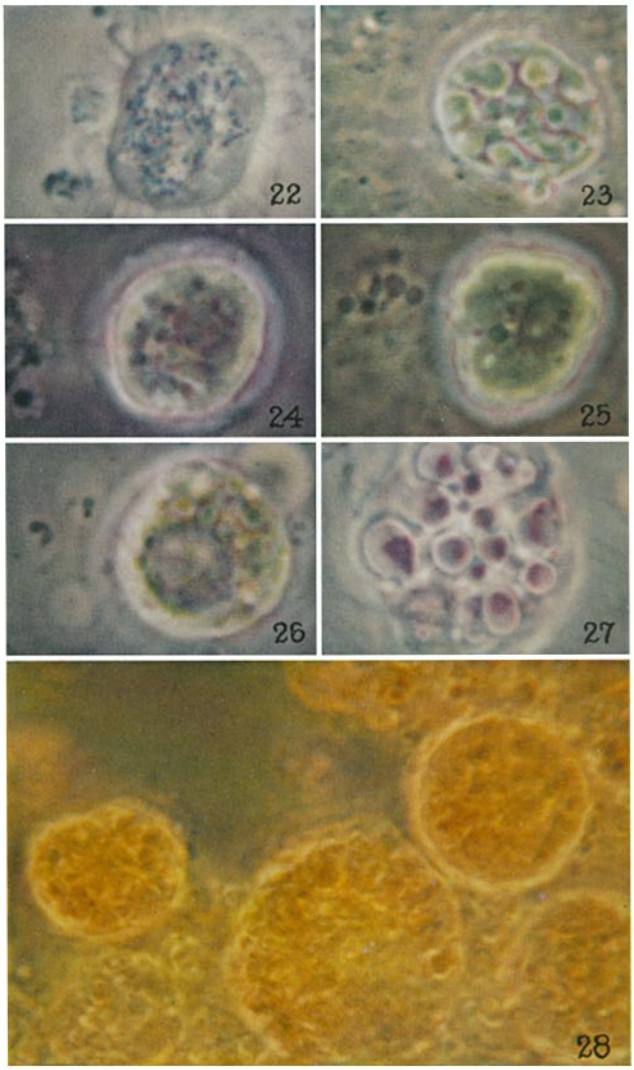

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PLATE 24

The disappearance of the protein droplets.

FIG. 29. Droplets 24 hours after the intraperitoneal injection of egg white proteins. Compare with 18 hours after injection (Fig. 7). There is no great decrease in the number of droplets, but they are much smaller and stain irregularly. Note the moth-eaten appearance of those indicated by the arrow. The general effect is similar to that observed in the immature droplets of Fig. 5. Calcium-formol fixation, Gram stain. × 960.

FIG. 30. A similar preparation of droplets 40 hours after injection of egg white proteins. A marked decrease in the number of droplets; those remaining are small. × 960.

FIG. 31. A similar preparation 72 hours after injection showing the average appearance of cross-sections through the proximal convolutions; only an occasional fine droplet remains. In the same section an occasional group of cross-sections through the proximal convolutions of a single nephron could be found in which more droplets had persisted. × 960.

FIG. 32. Cross-section of proximal convolution of an exceptional nephron 53 days after injection of egg white proteins fixed in Helly's solution and stained with iron-hematoxylin. The appearance of most of the proximal convolutions in this section was normal; *i.e.*, they contained their usual rodlets and no droplets. In this example, the droplets, which at 18 hours filled the tubules of all the nephrons, have disappeared and are replaced by rodlets with their original conformation, except at two places marked by arrows. × 960.

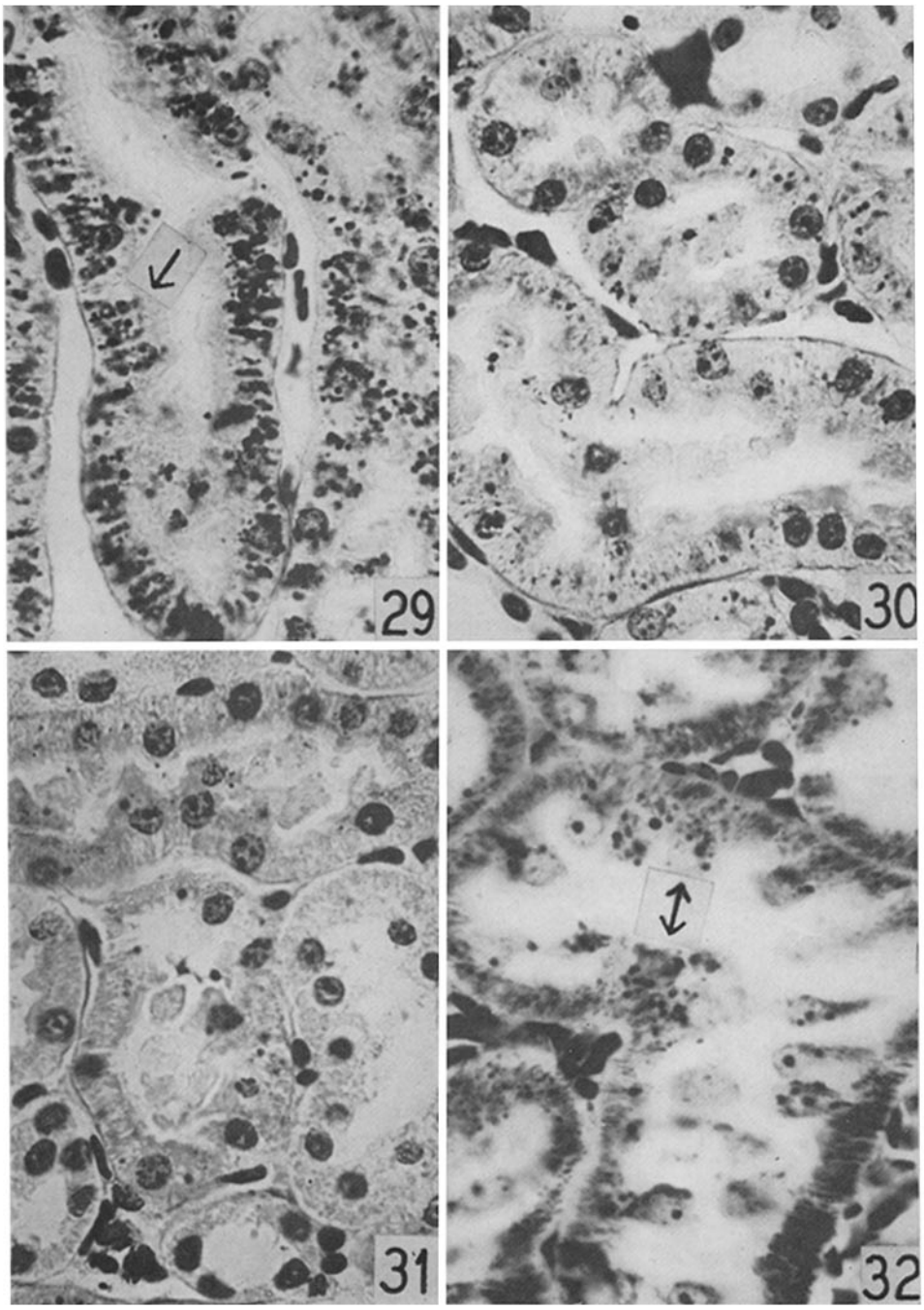

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PLATE 25

FIG. 33. Droplets 4 days after the injection of three daily doses of horse serum. Calcium-formalin fixation and Gram stain. × 960.

FIG. 34. A similar preparation 22 days after similar treatment, showing reduction in number and size of droplets. × 960.

FIG. 35. Similar preparation showing droplets 8 hours after the final of three daily injections of rat serum. × 960.

FIG. 36. Decrease in rat serum droplets on the 8th day following the last injection. Same fixation and stain. × 960.

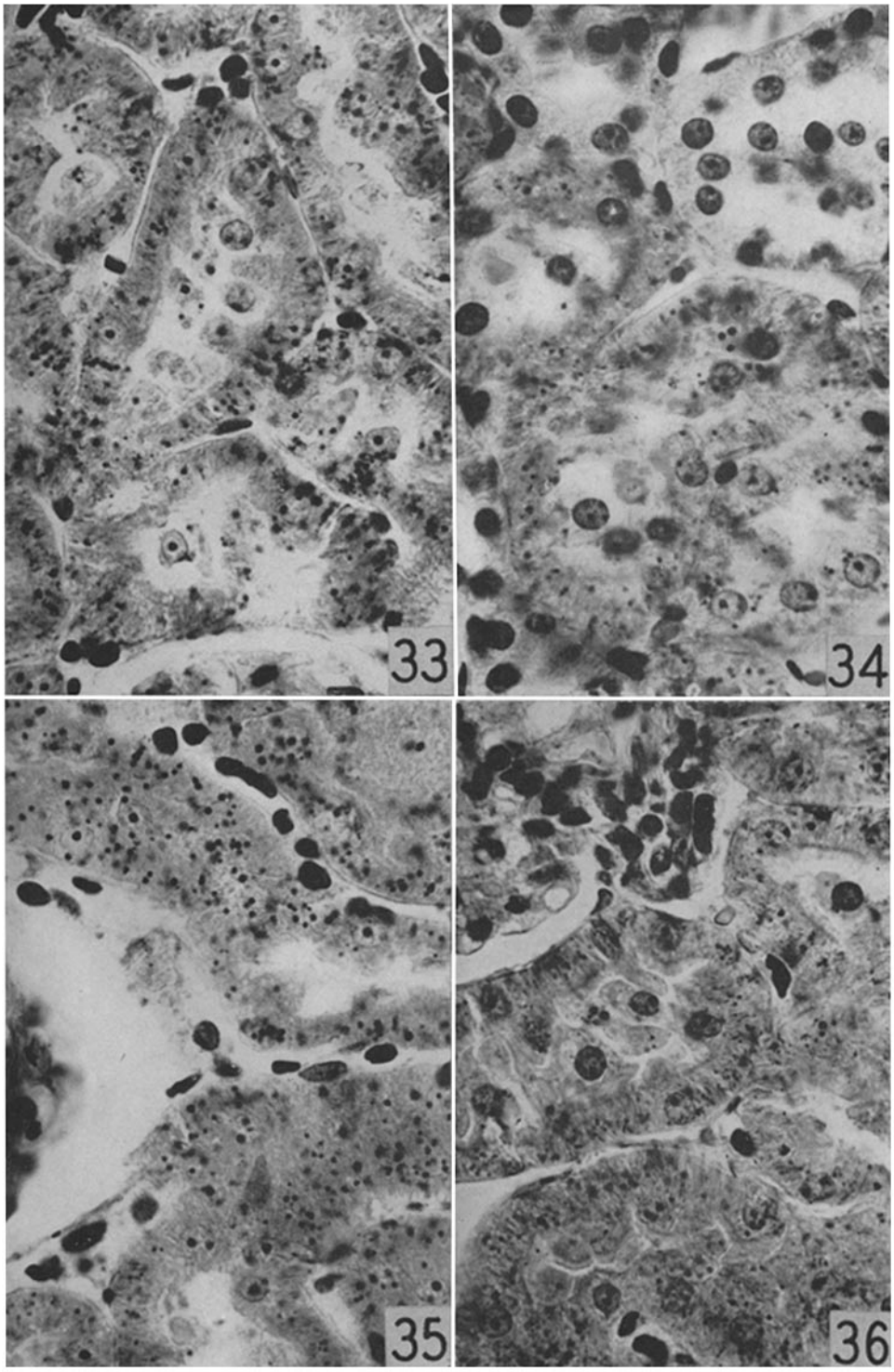

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PLATE 26

FIG. 37. Similar preparation of droplets of bovine albumin droplets 4 days after injection. × 960.

FIG. 38. Similar preparation 22 days after injection: a few fine droplets persist. × 960.

Disappearance of protein droplet during incubation of cells *in vitro*.

FIG. 39. Helly's fixation and iron-hematoxylin stain from an uninjected normal rat kidney after 30 minutes' autolysis at room temperature. In the cross-sections of proximal convolutions the rodlets have disintegrated to small round spherules confined to the basal portion of the renal cells.

FIG. 40. A similar preparation after 3 hours' incubation at 37° from the kidney of a rat injected 18 hours previously with egg white proteins. The few mitochondrial rodlets that may have persisted have disappeared, as a result of the autolytic processes, but the large protein droplets persist. × 960.

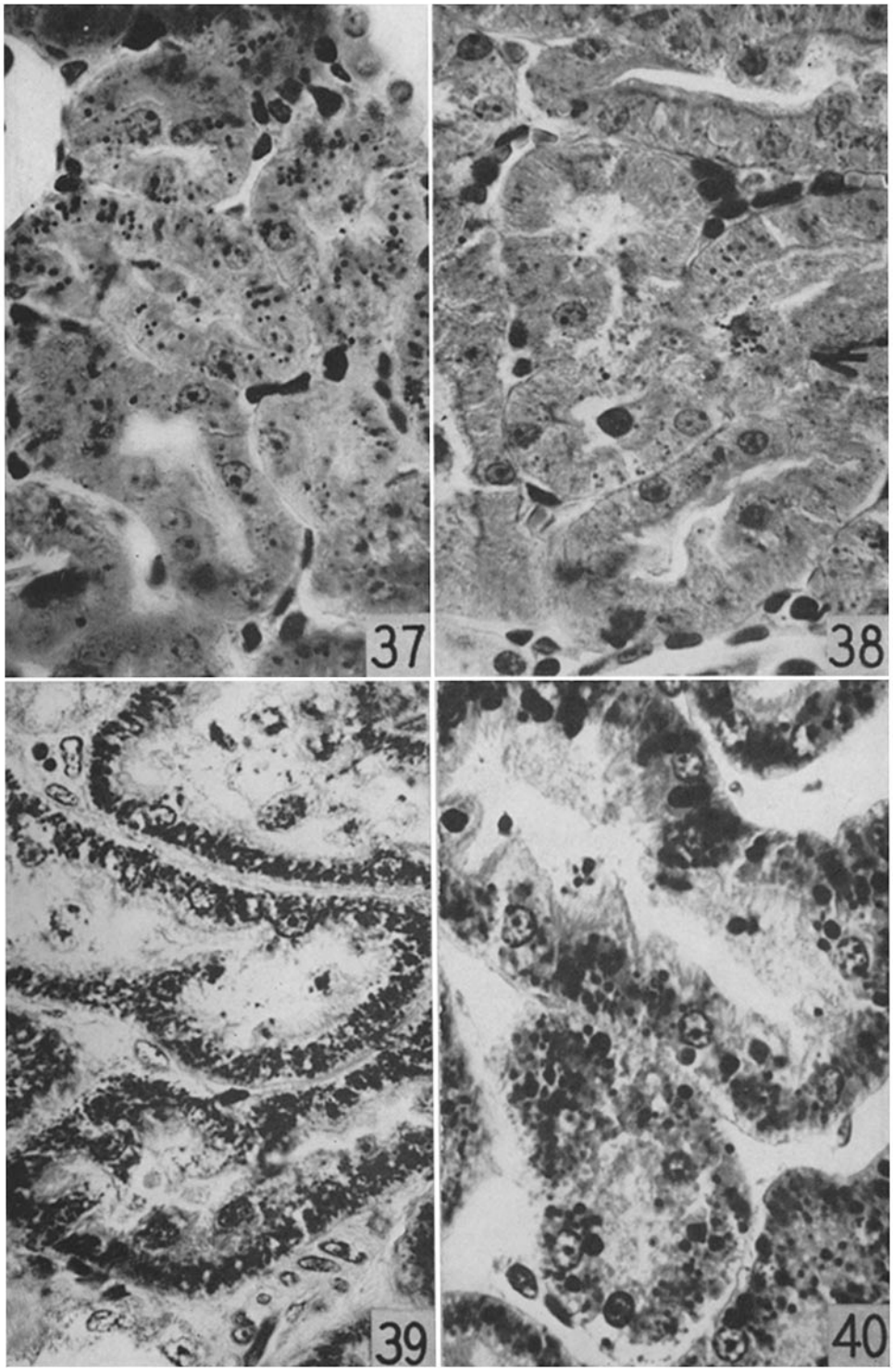

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PLATE 27

FIG. 41. Cortical tissue from the same kidney fixed in calcium-formalin and stained by the Gram method after 3 hours sterile incubation. Among the large deeply stained, well preserved droplets are others, smaller and staining irregularly. × 960.

FIG. 42. A similar preparation from the same kidney after 24 hours at room temperature. A marked destruction of droplets, with irregular staining and moth-eaten appearance. × 960.

FIG. 43. From the same kidney after 48 hours at room temperature. The droplets have disappeared. The tubule is greatly swollen, nuclei are pycnotic, the basement membrane persists. No bacteria are present. × 960.

FIG. 44. Autolysis of rat serum droplets. A similar preparation fixed immediately after sacrifice 8 hours after a third daily injection. × 960.

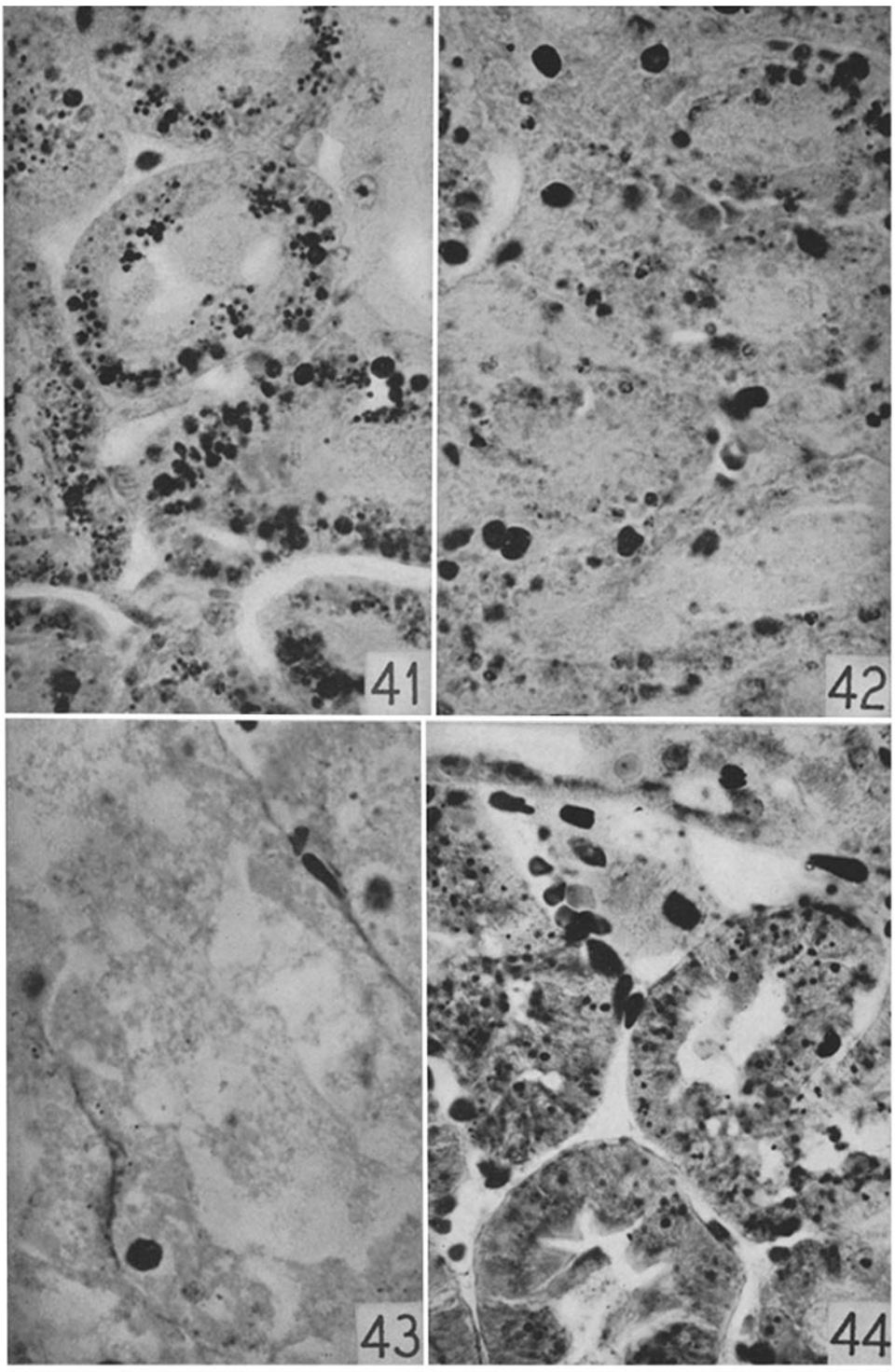

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PLATE 28

FIG. 45. From the same kidney after 1 hour incubation at 37°; only an occasional droplet persists. × 960.

FIG. 46. Crystallized ovalbumin droplets in tissue fixed immediately on sacrifice 18 hours after injection. × 960.

FIG. 47. From the same kidney, tissue fixed after incubation of 1 hour at 37°. × 960.

FIG. 48. Another portion of the same kidney after 3 hours' incubation at 37°; no droplets remain. × 960.

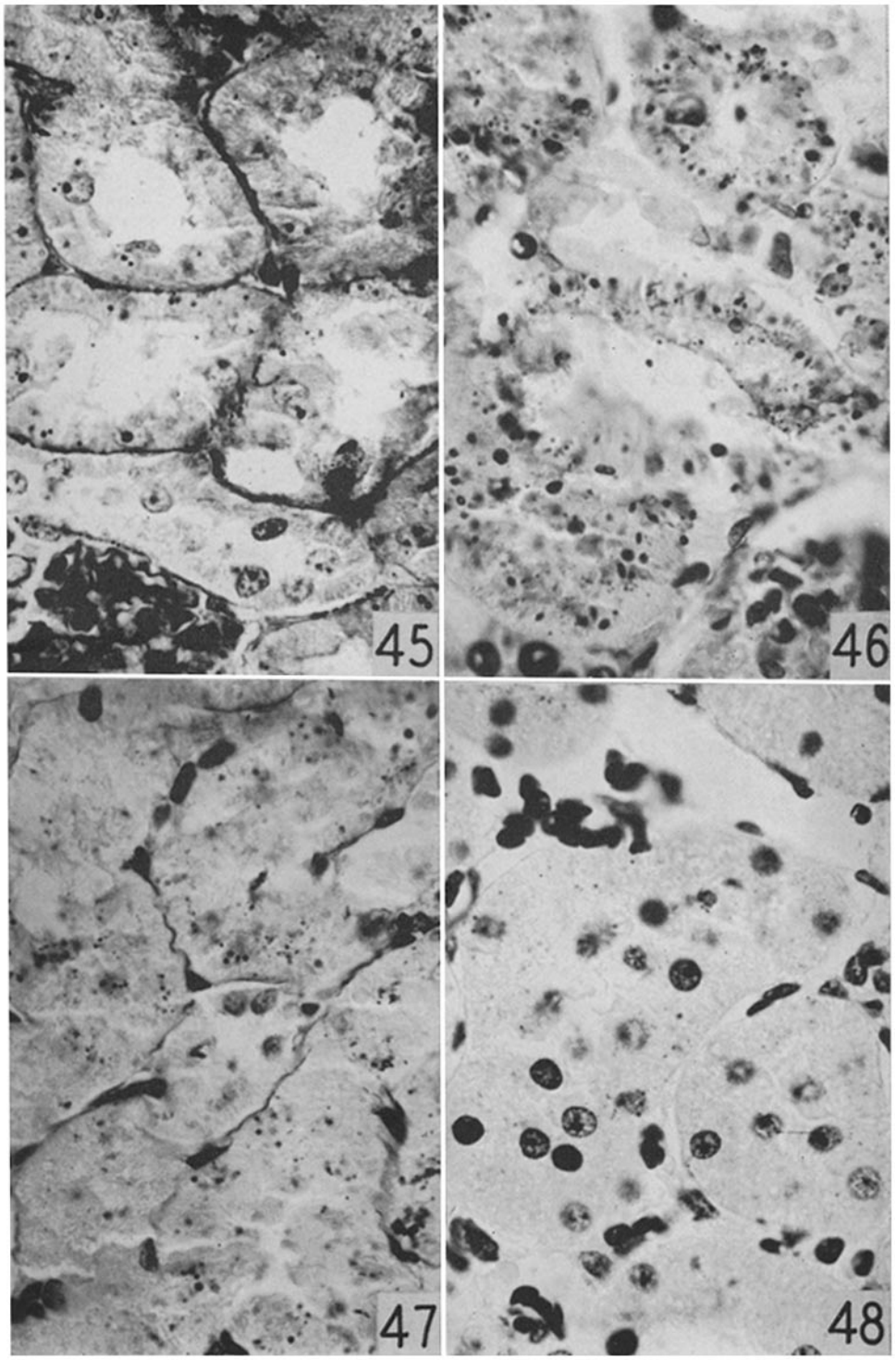

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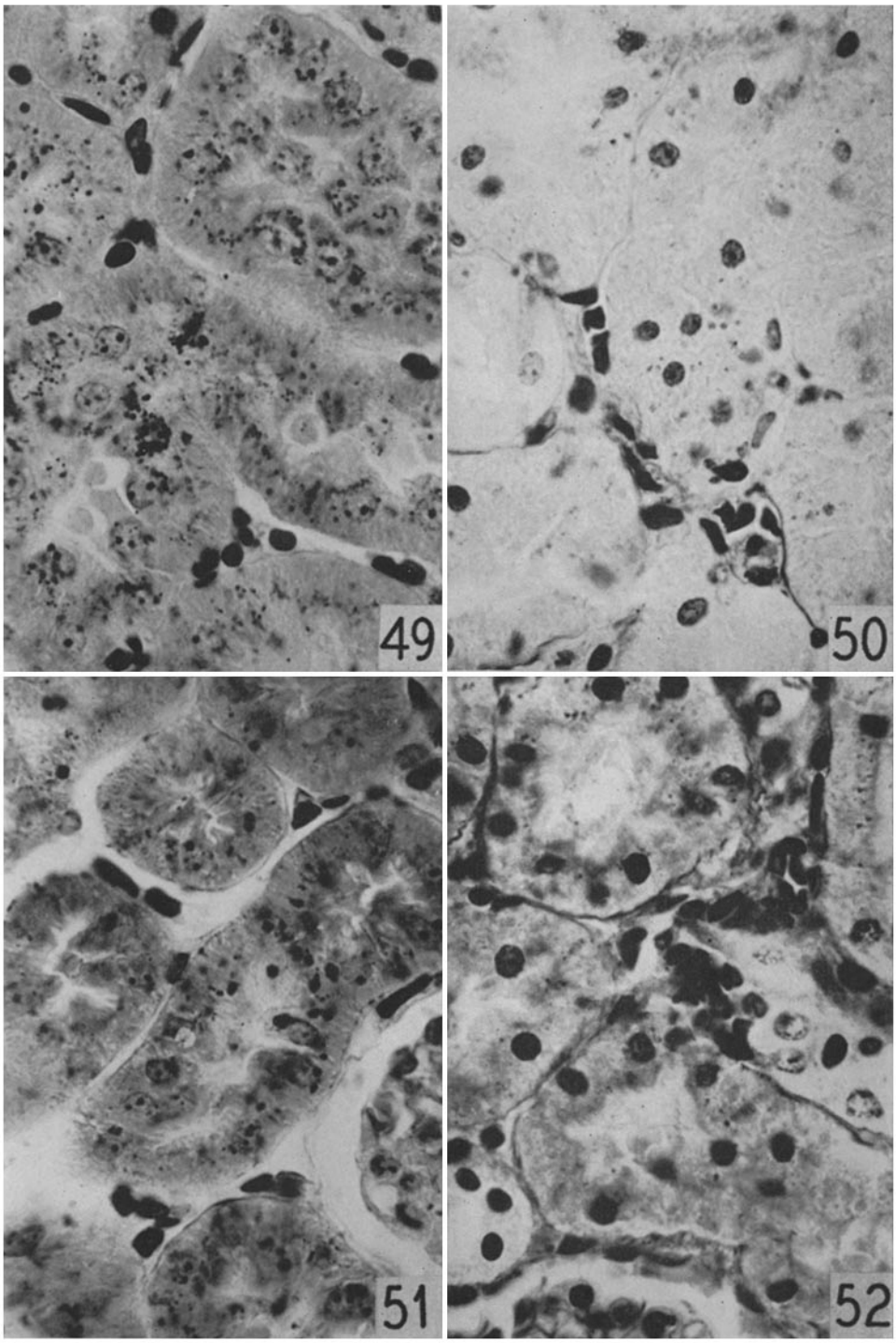
PLATE 29

FIG. 49. Bovine albumin droplets; immediate fixation 18 hours after injection. × 960.

FIG. 50. From the same kidney after 3 hours' incubation at 37°. × 960.

FIG. 51. Horse serum droplets; fixed immediately on sacrifice. × 960.

FIG. 52. From same kidney after 3 hours' incubation at 37°. × 960.



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