CELLULAR MECHANISMS OF RENAL SECRETION. A STUDY BY THE EXTRAVITAL METHOD

I. The Structural Phase of the Secretory Mechanism*

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It is evident from a survey of the voluminous literature of renal activity that a state of confusion exists in our knowledge concerning the secretory activity of the renal cells. In illustration of this fact we may call attention to an example of the conflict in conclusions and theories that prevails and examine briefly the reasons for its existence.

If one considers the relatively simple problem of the correlation between morphological alterations in the cells, specifically in the mitochondrial apparatus, and functional activity, a great discrepancy of opinion is found, ranging from the belief of such investigators as Kolster (1), Policard (2) and Oliver (3) that such changes may be correlated with varying functional states, to that of others, such as Cushny (4) and Emge (5) who are skeptical of any relation whatsoever between these two aspects of cell activity. General opinion is therefore quite properly unsettled.

Certain reasons for this condition are not far to seek in the light of present knowledge.

1. The basic difficulty has been that we have had no exact definitive knowledge of what part, if any, secretion may play in the elimination of substances into urine. Experiments have had to be designed on a hypothetical basis and have therefore proceeded not from the known but from the assumed. As an example of this, an increase in the volume of urine due to the intravenous injection of salt solution has been assumed to produce an increase of secretory activity. It is not surprising that the sought for structural changes in the renal epithelium were indefinite, when one considers that we know now that the increased urine eliminated under such conditions is best accounted for by the activity of other parts of the renal unit than the tubule.

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2. Another confusing factor whose importance has been recognized only in recent years is the part played by absorptive processes in the function of the renal cells, yet the operation of such processes would make it impossible to decide how much of any observed structural change might be due to the intake or to the output of material into the urine.

3. Another almost insurmountable difficulty has been always present in that the kidney to be examined in the experiment was already in a certain functional state whose possible effects had to be distinguished from those supposedly produced by the experimental procedure. Various means were taken to put the kidney at rest before the experiment, but these could in their turn only be based on the hypothetical assumption as to what normally produced secretory activity. Moreover, it is quite possible that some of these procedures, such as fasting, may have produced structural alterations in themselves, with a resulting further confusion in the experimental results.

4. The experiments of the past were all conducted on the living animal and in many cases considerable time elapsed during their course. It will be easily recognized that with such a responsible organ as the kidney the animal itself acts as a sort of independent and not necessarily cooperative agent in the procedure and the part it plays may well defeat the entire purpose of the experiment since the investigator is easily deceived by a change in its conditions that have thus arisen unknown to him.

5. Dependence in all the investigations of the past has been placed almost solely on morphological evidence. Granules were found in rows near rods and therefore were assumed to have arisen from the rods. It is obvious that the only accurate statement of such a conclusion is that it looks as if they might have done so.

That the writers believe these severe criticisms of previous investigations have arisen from the unsatisfactory state of knowledge of the times, will be evident when it is pointed out that all of them may be directed toward certain of the investigations of the senior author (3). But very considerable advances have been made, especially those which have come from the introduction of new methods, notably direct visual examination of the kidney as devised by Richards (6) and perfusion as adapted to the frog's kidney by Hoeber (7). The investigator is now able to again examine the problem in a new light.

The following pages describe an attempt made with what may be considered another new method. This method combines a morphological and a physiological examination of the activity of the isolated perfused kidney, using histological methods for the former purpose and a modification of Hoeber's method for the latter. As a result both the structural and functional aspects of the response of the tissues to

either physiological or pathological stimuli can be observed and correlated simultaneously. The application of the method to some of the problems of experimental nephritis has already been described and the term "extravital" suggested to describe it (8).

Another aid to our immediate problem has been found in the discovery that the dye neutral red is eliminated by the isolated perfused kidney almost entirely through the tubular epithelium and not in any significant amount through the glomeruli (9). Similar findings had been reported independently by Scheminzky (10) and recently the occurrence of its tubular elimination has been supported by the findings of Richards (11) that in the living frog the degree of its glomerular filtration is not adequate to account for its appearance in the urine.

The possible use of the extravital method in the problem under discussion for a study of the definitely proven secretion of neutral red is evident. By means of such a combination we can be certain that the tubule cells of a kidney eliminating neutral red are actually secreting, and we can also be certain that the dye found in the cell bodies has come from the blood vessel and not been absorbed from the lumen of the tubule. Absorptive process alone may also be produced by the new method in the functioning organ without any secretory activity, and the histological pictures then compared. The extravital method also avoids a further difficulty we have mentioned since the state of the kidneys before the experiment can be determined either by examination of one kidney before the perfusion is started or a portion of the kidney that is to be perfused may be examined before or at any stage of the experiment. So, too, any modification of the activity of the kidney on the part of the animal is prevented by the isolation of the organ. The nervous system is eliminated and all of the constituents of the fluid with which the cells are working are accurately known and these can be modified as desired as the experiment proceeds. The urine is collected almost simultaneously with the activity of the cells, rather than in long periods after its formation. And, finally, it is possible to examine the problem, placing little weight on a subjective interpretation of morphological evidence.

Technique

Histological Methods.—The following methods have been used for the morphological part of the work. For a general picture of the state of the tissues Zenker's,

Bouin's and 10 per cent neutral formalin in 0.9 per cent salt solution were used and as a stain Delafield's hematoxylin and eosin. For the preservation of the mitochondria and other granular structures of the cells Kolster's method of fixation was found most satisfactory. Bensley's fluid in our experience was less certain in its results. For staining the mitochondria and granules a method which is essentially the original Altmann procedure was used; this was supplemented by the Bensley method. For special purposes the Gram-Weigert stain was also used on tissues fixed by all the methods described above, and formol-Zenker fixation for the permanent fixation of neutral red in the tissues and subsequent embedding in paraffin and preparation of permanent sections (12).

Beside the common methods of fixation and staining other morphological procedures were used. The simplest of these was the examination of the fresh tissues. Small bits of tissue from the kidney were teased on a slide in Locke's solution and then crushed between cover-glass and slide with a twisting motion. Another valuable procedure in the morphological examination of certain problems was supravital staining with neutral red and Janus green. Vital staining of the kidneys with neutral red was also employed. The details of the use of these dyes will be given later.

Another method of staining the living tissue may be termed "extravital staining." The dye, either neutral red or Janus green, was added to the perfusion fluid after the normal function of the kidneys had been established and the cells of the tubules then stained themselves while actually functioning, a form of staining which can be called neither supravital nor vital staining.

Material from all the various vitally stained tissues was examined in the fresh condition as described above. Preparations from all were also fixed in formol-Zenker's and Kolster's fluid and permanent sections made from paraffin-embedded tissues.

The Perfusion Method.—The details of the method of perfusion of the isolated kidneys have been previously given (9). It is based on Hoeber's (7) modification of the Barkan, Broemser and Hahn (13) technique by which isotonic Locke's solution containing 0.025 per cent sugar and a small amount of glycocol maintained at a pH of 7.5 is perfused through the kidneys from separate containers by both the renal arteries and the renal-portal veins. The urine is collected from each kidney in a cannula. The urine formed by the procedure, when successful, is sugar-free, its electrolyte content is less than one-half that of the perfusion fluid and the rate of its excretion is comparable to that of the formation of urine by the living frog. If urea or dyes, such as phenol red or neutral red, are added to the perfusion fluid they are concentrated in the urine. The methods of determination of the constituents of the urine in the experimentation to be described were as follows: Benedict's method for sugar, electrolyte content by the Christiansen ionometer, the results being expressed as an equivalent per cent of NaCl, and dye content by the usual colorimetric methods.

As a basis for the consideration of the experimental findings we must first describe the appearances that are found in the functioning renal

cells of the living frog. The description that follows is essentially the same as that of Policard (2).

The Histological Characteristics of the Renal Cells of the Living Frog

Since the epithelial cells vary in their structure in different segments of the tubule the appearances seen in each are given separately.

1. The neck, or narrow portion I, of the tubule is lined with a low layer of cuboidal cells from which arise long actively motile flagella. The nucleus is small and round. The protoplasm contains a moderate number of granules which stain heavily with the acid fuchsin of the Altmann mixture. Very occasionally short thread-like structures may be seen among the round granules.

2. Segment II of the tubule is lined with a much thicker epithelium whose cells are covered with a brush border and whose nuclei are round or slightly oval.

Many different histological pictures are presented by the protoplasm of the cell. It may be filled with long tortuous filamentous structures often so closely packed together that little free protoplasm is seen, which run from its base to within a short distance of its apex. In such cells no round granules are found (Figs. 1 *a*, 1 *b*).

In contrast to the filamentous appearances just described the cell may show none of these structures, its entire protoplasm being crowded with large round granules that completely fill and even distend the cell body (Figs. 2 *a*, 2 *b*).

Between these two extremes all degrees of combination are possible. The general picture in the two kidneys of an animal is the same, though it may vary in different tubules and even between cells in the same cross-section of a tubule.

3. Segment III of the tubule connects the broad Segment II, or proximal convoluted tubule, with Segment IV or distal convoluted tubule. Its epithelium presents no distinctive characteristics but shows a gradual transition from the type described for Segment II to that which will be described for Segment IV.

4. Segment IV, or distal convoluted tubule, is lined with cuboidal cells with oval nuclei and which possess no brush border. Their protoplasm is distinguished by the presence of short thick rodlets which run almost the entire length of the cell. Granules are rarely found among its protoplasmic constituents (Figs. 3 *a*, 3 *b*).

That the filaments and granules of Segment II as well as the rodlets of Segment IV are preexisting structures and not the result of the action of the fixative solution may be easily shown by the examination of fresh tissues from the kidney in Locke's solution. The large round granules appear as highly refractile droplet-like objects while the filaments, though much less clearly seen than in the fixed material, are easily discernible as delicate striations or as an indefinite stippling of the protoplasm. The rodlets of Segment IV are more clearly outlined as stout refractile rodlets.

The findings in normal frogs living under physiological conditions may be summarized as follows: Marked variation in the condition of

the protoplasmic structures is seen only in Segment II of the tubule. The difference in the appearance of these cells is due to a change in the relative amounts of filamentous and granular material present in the cells. Our problem now is to connect if possible this structural variation with some variation in the functional activity of the cells.

The first step in such an attempt is to determine if the changes are concomitant with some functional state. Two such states must be considered; first, absorptive activity, which includes the absorption of the normal constituents of the glomerular filtrate, such as water, sugar and salt; and secondly, secretion, as may be evidenced by the elimination of neutral red by the cells.

The Absence of Effect of Certain Absorptive Processes upon the Filamentous and Granular Material of the Cell

It is possible by means of the extravital method to cause a kidney to function so that no secretory processes are involved in its activity though absorptive processes are actively at work. If the kidney is perfused with a Locke's solution which contains only simple salts and sugar, such a condition is established, for abundant evidence has shown that these substances are eliminated as a filtrate from the plasma by the glomeruli in about the same concentration as exists in the plasma or perfusion fluid.

A frog was pithed and prepared for perfusion in the usual way except that the left kidney was removed in such a manner as to leave all the cut vessels ligated. It was then fixed in the usual reagents. The other kidney was now perfused for 1½ hours with Locke's solution. Table I shows the functional findings. It will be noticed that from the urine about two-thirds of the total salt content and all of the sugar of the perfusion fluid had been removed. Other tests would have shown that water as well had been absorbed.

When the histological appearance of the right perfused kidney by the Kolster-Altmann method was compared to that of the left or unperfused organ, no differences in the granulofilamentous material were found. In the broad Segment II of both the unperfused (Figs. 4 *a*, 4 *b*) and perfused (Figs. 5 *a*, 5 *b*) organ the cells were filled with long tortuous filaments which extended from the basal membrane to the apex of the cell. Few granular bodies could be found. The cells of the Segment IV were filled with their short rodlets in sections from both kidneys (Fig. 5 *b*). The neck of the tubule and the glomeruli showed no significant differences except for the presence of red blood cells in the capillary loops of the tufts of the unperfused kidney.

This experiment shows the typical findings in kidneys whose granulofilamentous material of Segment II was in a filamentous state before the perfusion began. In repetitions of the experiment various conditions were found in the kidney removed before perfusion. In some there were no filamentous structures at all in the cells of Segment II, and these were filled and distended with large round granules. Yet the rodlets of Segment II were still visible and these cells contained few or no granules. In other experiments combinations of filaments and granules in varying amount were seen in Segment II before perfusion In all these cases the unperfused kidney showed exactly the same arrangement of the stainable material, whether filamentous or granular, in the cells of Segment II.

TABLE I

Absorptive Processes Only in the Kidney

Time	Arterial flow	Venous flow	Urine volume	Salt in perfusion fluid	Sugar
	cc. per hr.	*cc. per hr.*	*cc. per hr.*	*per cent*	
10:45–11:00	680	880	3.6*	27	0
11:00–11:15	720	800	5.6	39	0
11:15–11:30	760	840	4.8	38	0
11:30–11:45	760	840	6.0	40	0
11:45–12:00	720	800	5.2	38	0

* Calculated as output of two kidneys.

It is reasonable to infer two important facts from these experiments. First, that the method of perfusion does not in itself affect the histological appearance of the filamentous granular material. And second, that the processes of absorption, in as far at least as they concern the important urinary constituents of salts, water and sugar, can be eliminated as a possible cause of any morphological changes that may be found in the granulofilamentous structures of the cells of Segment II.

The Effect of a Secretory Process on the Granulofilamentous Material of the Renal Cells

The evidence which indicates that neutral red is secreted by the tubule cells of the kidney has been previously mentioned (9–11). In the following experiment the effect of this secretory process on the protoplasmic constituents of these cells is demonstrated.

The frog was prepared for perfusion and a normal urine was formed as shown in Table II. After one sample had been collected the left kidney was removed and

fixed in the usual solutions, and neutral red in a concentration of 1.25 mg. per 100 cc. was added to the Locke's solution in the bottle supplying the tubular apparatus of the right kidney through the renal-portal venous system. There began an elimination of the dye which reached a rate of 1.0 mg. per hour in the third period. The right kidney was removed after two more periods and fixed. Sections of each kidney were stained by the Altmann method. The contrasting pictures are shown in Figs. 6 *a*, 6 *b* and 7 *a*, 7 *b*.

The left kidney which had been perfused with clear Locke's solution, and which had therefore no material to secrete, showed the filamentous appearance in the cells of the broad Segment II that has been previously described. Long tortuous threads filled the protoplasm and only very rarely could a definitely round granular object be found (Figs. 6 *a*, 6 *b*). The remaining divisions of the tubules showed the appearance that has been previously given.

TABLE II

Secretion of Neutral Red by the Kidney

Time	Arterial flow	Venous flow	Urine volume	Neutral red	Salt in perfusion fluid	Sugar
	cc. per hr.	*cc. per hr.*	*cc. per hr.*	*mg. per hr.*	*per cent*	
10:45–11:00	400	600	6.0	—	40	0
Left kidney removed						
1.25 mg. neutral red in 100 cc. Locke's solution to tubules of remaining kidney						
11:30–11:45	440	500	7.2*	0.63*	40	0
11:45–12:00	560	500	8.0	1.00	40	0
12:00–12:15	440	500	6.8	0.85	40	0
12:15–12:30	440	600	6.4	0.72	45	0

* Calculated as output of two kidneys

An entirely different picture was found, however, in the kidney which had been perfused with neutral red and which had secreted this substance into the urine. The dye by gross examination was seen to have evenly permeated the organ and sections of it showed a marked change from the appearances noted in the non-secreting kidney. In every cell of practically every Segment II no filaments could be found whatsoever, for the protoplasmic body was now distended with large round granules. In many instances they were crowded so closely together as to leave no intervening substance visible between them and so closely surrounded the nucleus as to obscure it (Figs. 7 *a*, 7 *b*). Yet in those sections where this granular appearance in Segment II was most extreme the cells of Division IV were still filled by their short thick rodlets (Figs. 8 *a*, 8 *b*). The portions of the neck and of Division III contiguous to Segment II showed a greater or lesser similarity to it.

As in the previous series of experiments with clear Locke's solution so in the tests in which neutral red was secreted, animals were found whose left kidney, perfused only by the dye-free fluid, showed a greater or lesser degree of the granu-

lar state in Segment II of its tubule instead of the purely filamentous appearance. The functional activity of these kidneys under the perfusion differed in no way from that of the preceding experiment. The appearances in Segment II of the non-secreting and secreting kidney were, however, found less dissimilar than those described in the preceding experiment. If the cells of the first kidney, non-secreting, were filled with granules and contained no filaments, then the second, secreting, was identical in appearance. If, however, some filaments were present in the first kidney, a dissimilarity was found, for the cells of the second kidney which had secreted the dye contained none of these structures but were filled with granules alone. As an example, the appearance of Segment II is shown in Figs. 9 *a*, 9 *b*, 10 *a*, 10 *b* and 10 *c*.

The experiments with neutral red just described allow the definite conclusion that in contrast with the negative effect of absorptive processes there occurs concomitantly with the secretion of neutral red a change in the histological appearance of the cells of Segment II that is characterized by the disappearance of filaments and the appearance of granules. If the cells are already in the granular form before the neutral red is given, then no further change occurs in the protoplasmic structures with the secretion of the dye. Secretion of the dye is therefore accompanied by structural changes in the renal cells that are identical with those noted as a part of the vital activities of the living animal and secretory activity occurs only with the histological picture of granule formation.

With the evidence at hand we are justified in stating that the change from the filamentous to the granular state is concomitant with secretory activity. Can a more intimate relation between the morphological and functional aspects of cell response be demonstrated so that we can definitely conclude that the functional activity is determined by the structural change? There remain also several questions of detail. Of the protoplasmic structures of the renal cell, what is mitochondrial substance and what is not? Are the granules and filaments independent structures or are they related in their constituent material or by a common origin? Are there various sorts of "granules" involved in the histological picture? Are the granules solid substance or are they of the liquid nature and therefore best considered vacuoles? All these questions will be examined directly and objectively by the method of extravital staining and the findings compared with those obtained by the other vital staining and fixed tissue methods.

The Changes Produced in the Isolated Organ by Extravital Staining with Neutral Red

Since an important part of the present study is a comparison of the findings by different methods of staining, material from the same experiments described previously, in which the appearance demonstrated by the use of the Altmann method has been noted, was also examined as examples of extravital staining.

In the experiment of Table II the remaining kidney at the end of the perfusion was found to be evenly stained by the neutral red which it had been secreting. The organ was a deep mahogany red in color, striped by the faintly pinkish bands of its connective tissue and vessels. It was not swollen nor edematous. Small bits taken from various portions of the organ were teased apart and crushed with a drop of clear Locke's solution under a cover-glass. In such specimens the dye was contained in its greatest amount in the cells of Segment II. From here the distribution continued, depending on the degree of staining, towards the neck of the tubule and downwards towards Segment IV with its rodlet cells. The former might contain a considerable amount of dye; the latter was free except for such diffuse staining as may be seen in any tissue that has been long in contact with a dye. The glomeruli contained no dye except for an occasional isolated cell of the clasmatocyte group within the tuft whose irregular protoplasm was filled with dye vacuoles.

The dye, in whatever epithelial cells were examined, was in the form of round granules. In slightly filled cells these were scattered indiscriminately through the protoplasm but in densely filled cells so packed the protoplasmic body as to completely obscure its detail. The nucleus lay as a clear round or oval area in the center of these clusters of granules and was unstained (Figs. 11, 12).

The dye may also be studied in paraffin sections from tissues fixed in formol-Zenker's and the same observations made.

The term "granule" has been used to describe the round deeply staining structures chiefly because that term seemed most appropriate for the description of appearances in the fixed tissues stained by the Altmann method. To most, the word "granule" holds the connotation of solidity and the term "vacuole" might therefore seem more appropriate for the appearances noted in the extravital preparation. That they are not vacuoles in the sense that they are cavities in the protoplasm filled with free liquid dye is shown, however, by the fact that when the cell is crushed they remain intact and float through the surrounding Locke's solution in which the cell is suspended. They remain discrete indefinitely and show little tendency to coalesce, their appearance being that of small droplets of red-stained semifluid material. For we may be sure that they are not solid substance by means of a change similar to one described in vitally stained animals by Policard (2) that occurs in them as the preparation stands. This consists in the

development within the granule of a smaller granule, a minute point-like or comma-shaped object which has all the appearance of condensed dye that has precipitated out of the dye content of the larger granule. The smaller object is usually affected by Brownian movement and may be seen twisting and turning within the larger granule, colliding with its inner surface and rebounding into the center again. This active movement proves that the substance of the granule proper must be at least semifluid, so, with our previous determination that its substance is immiscible with water and from what we know of the solubility of neutral red, the assumption that the granule is a droplet of lipoid material becomes extremely plausible. In one sense, therefore, the structure is a vacuole but in order to maintain the concept that fits the appearance in the fixed tissues so well and which has been used almost entirely by those who have previously studied such material we shall use the terms, "granule," "vacuole" and "droplet" as synonyms.

The question now presents itself as to the exact relation of these extravitally stained vacuoles and the large round granules which have been described in the Altmann preparation of fixed tissue from the extravitally stained kidney. Since we know from the examination of fresh tissues that the granules of the fixed tissues are preexisting objects a glance at Figs. 7*a*, 7*b* and 11, 12, which are from the same kidney stained by the two methods, will show that the extravitally stained objects and the Altmann-stained granules are certainly the same formations. Apart from the similarity of their appearance there would not be space for two preexisting different sets of such structures to exist side by side within the limited room of the cell body. The granule of the extravitally stained kidney can therefore be considered a semifluid lipoid droplet deeply stained with neutral red which when fixed is preserved and stainable by the fuchsin of the Altmann method. It would seem reasonable to extend this conclusion to include in the same category the unstained granules seen in cells of the living animal which resemble identically by the Altmann method the objects seen in the extravitally stained neutral red experiment. Supporting evidence of this identity will appear later.

In the description of the kidney stained extravitally with neutral red there has been no mention of filaments within the cells. It is true that these structures have disappeared, at least in their long thread-like form, with the occurrence of secretory activity and the development of the granules but, as previously described for the Altmann preparations, portions of them always remain in the lower part of the

cell, clustering around the nucleus where they appear as short bacillary or vibrio-like objects. In neutral red extravital preparations they are never seen, for here all the stained objects are the definitely round large granulovacuoles. That these thread-like structures are indeed present and that they too may be stained extravitally is shown, however, in the next series of experiments.

The Changes Produced in the Isolated Organ by Extravital Staining with Janus Green

The method of extravital staining with Janus green was identical with that used with neutral red. The perfusion was begun with clear Locke's solution and after one or two samples were examined to determine if a normal condition of kidney

TABLE III

Action of Janus Green on the Kidney

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	*cc. per hr.*	*cc. per hr.*	*mg. per hr.*	*per cent*	
10:00–10:15	400	500	6.0	—	45	0
1/500,000 Janus green in Locke's to tubules of remaining kidney						
10:30–10:45	400	450	6.0	0	45	0
10:45–11:00	450	400	8.0	0	40	0
11:00–11:15	500	400	10.0	0	45	0
11:15–11:30	450	450	7.2	0	45	Tr.

function had been established, the dye was added to the fluid in the bottle that supplied the tubular circulation. Janus green is a definitely toxic substance, so that a dilution of 1/500,000 was used. Another advantage of this dilution whose importance will be emphasized later is the specificity of its staining reaction. But even with such a solution the kidneys showed evidences of damage after 2 or 3 hours of perfusion so that the definitive experiments were limited to 1 hour or less.

The results of such extravital staining with Janus green are shown in Table III. It will be observed that there was no serious disturbance in the function of the organ, but the dye was not excreted. An explanation of this lack of secretion will be considered later.

In the gross, the kidneys were unchanged except for their dark slate-green color. When examined in fresh crushed specimens many of the cells of Segment II appeared similar, save for color, to those from the neutral red kidney. Other cells contained the large green round granulovacuoles but when examined with the oil immersion a striking difference from the earlier preparation with neutral

red was noted. For besides the large round green granules there were found in those parts of the cell where the protoplasm was not occupied by them fine thread-like dark green bacillary objects (Fig. 13). Another difference from the neutral red staining was noted in Segment IV. In the Janus green extravital preparations the short thick rodlets were definitely stained distinct green. There were, however no large green vacuolar formations in this division of the tubule.

The staining of the thread-like bodies with Janus green together with their reaction with bichromate and their appearance when stained by the Altmann method leaves no doubt that these objects can be considered mitochondria in the strict and modern sense of the word. The same is true for the rodlets of Division IV. That these filamentous mitochondria have disappeared in great part from the cell bodies of Segment II of kidneys actively secreting neutral red and that they only persist in those portions of the protoplasm that are not occupied by the large droplet formations is worthy of especial note.

The nature of the large droplets in the Janus green extravital preparations is less certain, however, for they have all the appearance, save color, of the objects seen in the kidney stained extravitally with neutral red, and mitochondria do not stain with this dye. Our next problem must be, therefore, to determine whether these structures in the Janus green preparations are indeed the same objects seen in the neutral red preparations.

All the morphological similarities we have mentioned are strong presumptive evidence that the large round green bodies of Janus green extravital staining are identical to the red ones of neutral red extravital staining. Still one might demur to such a conclusion with the suggestion that in the former case the droplets have arisen as a result of some specific or toxic action of the Janus green. The extravital method allows us, however, to answer this objection in a decisive manner for it can be shown conclusively that the two appearances are the same object stained by one or the other dye.

The Changes Produced in the Isolated Kidney by Combined Extravital Staining with Janus Green and Neutral Red

Since neutral red is a more rapidly acting dye than Janus green the latter was perfused first to the tubule of the kidney in a dilution of 1/400,000 in the manner previously described. Table IV shows the results. For 1 hour a urine normal except for the appearance of faint traces of sugar was obtained. The bottle con-

taining the Janus green solution that had been supplying the tubules was now replaced by one containing Locke's solution with neutral red in a concentration of 1.25 mg. in 100 cc. and the perfusion was continued for three more 15 minute periods with no serious impairment of any function save that the elimination of neutral red was markedly depressed. The significance of this striking difference from the profuse elimination of neutral red in the previous experiments can be discussed more clearly after the results of the experiments of the following paper have been described. The organs were now examined and appeared grossly normal except for their muddy brownish red-green color.

TABLE IV

Action of Janus Green and Neutral Red on the Kidney

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	*cc. per hr.*	*cc. per hr.*	*mg. per hr.*	*per cent*	
10:45–11:00	400	760	4.0	—	40	0
	1/400,000 Janus green in Locke's to tubules					
11:00–11:15	400	760	4.8	0	45	0
11:15–11:30	400	800	3.6	0	40	0
11:30–11:45	360	760	3.6	0	50	Ft. tr.
11:45–12:00	400	760	3.2	0	45	Ft. tr.
	1.25 mg. of neutral red per 100 cc. Locke's to tubules					
12:00–12:15	400	740	4.8	Tr. n. red		Ft. tr.
12:15–12:30	400	700	4.0	Tr. n. red	45	—
12:30–12:45	400	760	4.4	Tr. n. red	40	Ft. tr.

A low power examination of fresh crushed specimens showed at once that there had occurred a mixed staining of the tissues with the two dyes. In some of Division II the cells were uniformly filled with either green or with red granules and in the former the bacillary mitochondria could be seen. In other tubules there was a mixed staining of individual cells. In some were red and green droplets in varying proportions indiscriminately scattered throughout the cell body. In such cases each droplet seemed to be stained purely with one or the other dye (Fig. 14). Others though showing pure red and pure green granules also contained some of these bodies definitely stained with a mixture of the two dyes which gave them a purplish slate-green color (Fig. 15). With all such appearances the fine green mitochondria might be found. Even the picture shown in Fig. 16 was observed where in a protoplasm filled with many green mitochondria only a few large neutral red droplets were present.

The varied pictures seen in these preparations have but one interpretation; namely, that the granulovacuolar bodies which were

stained indiscriminately by either Janus green or neutral red or by both in mixture are in fact the same structures. It is a fact of considerable significance as will appear later that these structures stain so readily in a very dilute solution of Janus green.

Our experiments have demonstrated one definite difference in the reaction of the mitochondria and the granulovacuolar bodies, since the former never stain with neutral red. Another reaction has been found which indicates a difference in the constitution, either physical or chemical, of the two structures, and that is their reaction to the Gram stain. In all specimens, whether from animals living under natural conditions, from kidneys perfused with plain Locke's or from those extravitally stained with either neutral red or Janus green, the granulovacuolar bodies retain the Gram stain (Figs. 23–25). Such Gram positivity, however, is never present in the mitochondria, either in the fine bacillary forms and long filaments of Segment II or in the short rodlets of Segment IV (Fig. 26).

Another difference between the vacuoles and the mitochondria that can be demonstrated by means of the Gram-Weigert stain is the fact that the former do not need to be bichromatized to resist solution in alcohol as do the mitochondria, and also that they resist the action of acetic acid in the fixing solution, a substance which completely destroys all evidence of mitochondrial structures (Figs. 23, 24).

Again the question may arise if we are dealing with the same granulovacuoles in these sections stained with Gram as in the preparations stained by the extravital method.

A Gram stain done on tissues after such treatment in which the neutral red has been preserved by fixation in formol-Zenker's solution shows cells containing blue vacuoles, red vacuoles, a mixture of the two and granules whose color is a purplish combination of red and blue (Fig. 17).

As stated, it is our plan to bring to bear as many different methods as are applicable to the problem in order to confirm our contention that the extravital method not only produces no artifacts in the tissues that are being examined by it, but that under its conditions processes of the same nature may develop as actually occur during life. For these reasons supravital and vital staining with the two dyes was done and the results compared with those of extravital staining. It is of

course impossible to perform elaborate tests with these less controllable methods. Enough of the salient features of the previous experiments can be observed with them however to afford conviction that the processes that occur in the handling of the dye are similar under the conditions that obtain in all three methods, vital, supravital and extravital. The findings that we give are in a summary form.

Appearance of the Supravitally Stained Renal Epithelium

Supravital Staining with Neutral Red.—Bits of fresh kidney tissue from killed animals were teased and agitated in a concentration of 0.25 mg. neutral red in 100 cc. Locke's solution. At intervals small portions were removed and examined in fresh crushed smears with Locke's solution. The appearances in the cells can be summarized by the statement that they are identical with those we have described as a result of the extravital method. A few minutes after being placed in the dye solution red granules appeared in the cells of Segment II of the tubule which increased in number with time, so that in a short period, whose length depended apparently chiefly on the speed with which the dye could permeate the teased tissues, the protoplasm of the cells was filled with large round droplets that surrounded the unstained nucleus (Fig. 18). There was no staining of the mitochondria nor were the rodlets of Division IV colored. This latter portion of the tubule was also free of the larger round granulovacuoles. In the vacuoles, especially in the older preparations, the same small motile granule within the larger one was seen. In other words one cannot distinguish a fresh crushed specimen of such a preparation under the microscope from one from the previously described extravitally stained kidney.

Supravital Staining with Janus Green.—Bits of kidney tissue placed in Locke's solution containing a 1/500,000 concentration of Janus green showed in the course of a few minutes dye within the protoplasm of their cells. The poor penetration, however, of Janus green as compared to neutral red was particularly noticeable in the supravital preparations. Only the cells of the tubules on the periphery of the tissue masses were stained and these irregularly. The protoplasm of the cells of Segment II was filled with the fine bacillary mitochondria and among these were scattered large round green droplets in greater or less number. As time passed the latter increased in number while the mitochondria decreased (Fig. 19). In the vacuoles there developed the small motile granule and the rodlets of Division IV of the tubule were stained a definite green. In specimens that had stood for some time evidences of damage appeared. These consisted of irregularities in size and shape in both the mitochondria and the droplets and a greenish discoloration of the nucleus associated with pycnosis.

Combined supravital staining with the two dyes was also done and the appearances noted were entirely the same as in the combined extravital experiment. The double staining was accomplished by staining the tissues in mixtures of the

two dyes or successively in one and then in the other. The results depended on the difference in the relative permeability of the two dyes and by varying the procedure all the previously described pictures of extravital staining were produced. Some cells contained only large droplets of red, others only green droplets, while still others showed scattered granules of both colors and even granules whose color was a purplish green mixture of the two dyes (Fig. 20). Wherever the Janus green had entered the cell the mitochondria might be found in a greater or less amount depending on the number of droplets of one color or the other that were present. Perhaps the most striking figures were those in which red droplets were scattered through a protoplasm that still contained a considerable amount of green mitochondrial material (Fig. 21).

It is evident from these experiments that the processes involved in extravital and supravital staining are essentially similar. It remains to determine if the same identity obtains in the case of isolated tissues and of the processes that occur in the living animal secreting the dye. Comparisons were made with vital staining of living frogs.

The Appearances Noted in Vital Staining of Living Animals with Neutral Red

10 cc. of a 0.25 per cent solution of neutral red in Locke's was injected into the dorsal lymph sac of a living frog. The injection was repeated at hourly intervals three times and the animal killed 1 hour after the last injection. All of the fluid was absorbed. The tissues of the animal were now definitely pink, the coloration being especially noticeable beneath the skin and in the nucleus and oviducts. The urine in the bladder was pink. The kidneys were more or less heavily stained, some having the dark mahogany-red appearance described in the extravital experiment. The microscopical appearance of fresh crushed preparations from them was also identical to those of the extra- and supravital preparations. The cells of Segment II contained typical droplets, some in few numbers while others were completely filled and distended by them. In some kidneys whose cells contained only a few red granules there could be seen uncolored droplets. As the preparation stood the condensed motile particle of dye appeared within the vacuole. In cells which contained only a few vacuoles, it was possible to observe in the protoplasm free of these structures an indefinite unstained stippling whose detail it was impossible to resolve. When such vitally stained neutral red material was restained supravitally with Janus green, it was found that the stippling had represented uncolored mitochondria (Fig. 22) and as the supravital staining continued, typical large round vacuoles of Janus green appeared.

The experiments demonstrate the same structures and processes in the cells of living animals secreting the dye that were observed in the

extravital experiments. It follows that in the present instances the results of vital, extra- and supravital staining were essentially similar in their nature.

DISCUSSION

A summary can now be made of the structural changes that accompany secretion in the cells of Segment II and their significance can be appraised.

The following facts have been established.

1. Filaments and granulovacuoles are preformed protoplasmic elements of the renal cell.

2. During the normal life of the animal the cells of Segment II show a wide variation in the relative amounts of these two protoplasmic structures. In any given cell there is a converse relation between the amount of filamentous and granular material.

3. The principal absorptive processes that occur in the formation of the urine are not accompanied by variations in these structures.

4. The extravital secretion of neutral red which has been shown to be identical in its processes with vital secretion, is accompanied by a disappearance of filaments and the formation of granulovacuoles. If the cells originally contain only the latter, no histological alteration accompanies the secretion of the dye.

5. The granulovacuolar structures stain with neutral red during the secretion of the dye. This can be demonstrated either extravitally or vitally.

6. The filamentous structures never stain with neutral red.

7. The filaments do stain with Janus green. They also require bichromatization for their preservation and are soluble in weak acetic acid.

8. The granulovacuolar material also stains with Janus green even from weak concentrations of the dye but does not require bichromatization and is not soluble in dilute acetic acid.

9. The filaments are Gram-negative, the granulovacuolar structures Gram-positive.

That the structural changes observed as concomitant with the secretion of the dye play a part in the actual secretory process seems certain when one considers that the dye is concentrated within the cell in the

vacuolar structures during the course of the secretion. The significance of this concentration is examined in an accompanying paper. That the filaments are mitochondrial is also obvious. The question remaining for determination is that of the nature of the vacuoles and of what relation they bear to the mitochondria. From a consideration of the facts noted above, two interpretations are possible.

1. The filaments and granules are entirely independent structures that bear no relation to each other. On this hypothesis the mitochondria play no rôle in the secretion of the dye. But if such is assumed one is at once confronted with the difficult admission that highly diluted Janus green is not so specific a stain as a long series of investigations would indicate. There also remains without explanation the mysterious disappearance during the secretory process of the filaments which one must assume have vanished leaving no trace while at the same time an equally unexplained appearance *de novo* of granulovacuoles has occurred, derived not from the mitochondria, but from an unknown substance which has the generally recognized characteristic staining reaction of mitochondria.

2. The granulovacuoles are derived from the mitochondrial filaments by alteration both in their form and constituent substance. The change in substance is of such nature that neutral red now stains them, leaving unaffected, however, the original staining reaction of this altered mitochondrial material to Janus green.

The second interpretation seems to us much the more satisfactory. It explains, for example, the converse occurrence of filaments and granules which is so constantly observed; the reaction of the vacuole to dilute Janus green as a remainder of the characteristics of the original mitochondrial substance; it indicates what becomes of the substance of the disappearing mitochondria and designates from what source the material of the originating granulovacuoles is derived. By it the mitochondria are made essential factors in the secretion of the dye, since the change which occurs in them is the source of the final mechanism by which the dye is concentrated within the cell body. The exact nature of this alteration which accompanies the change of the mitochondrial substance into vacuolar substance is not definitely shown by the experiments but the altered reactions of the latter to dyes and fixatives suggests that a splitting of the protein-lipoid com-

plex of the mitochondrial material has occurred, with a consequent liberation of its constituent substances, so that each, now no longer bound and inert, reacts in its characteristic manner. The freed lipoid takes up neutral red while the protein element, insoluble in alcohol without bichromatization, reacts with the Gram stain like fibrin.

One might visualize the course of events in the secretory process already described somewhat as follows. Neutral red enters the cell from the blood vessel. Owing to its presence and to factors as yet undetermined the mitochondrial filaments disintegrate. One can think of many forces that might cause such a result, such as changes produced by the presence of the dye in interface surface tensions, osmotic pressure or diffusion currents. From the material of the disintegrated filaments and as a result of constituent alterations, vacuoles are formed in which the dye being readily soluble is concentrated. Whatever vacuoles may be present from previous processes of secretion are also saturated with the dye. And here the description of the process of elimination must, for the time being, end, for no evidence whatsoever has been obtained as to how the dye passes from the vacuoles into the lumen of the tubule. This question will be examined in the succeeding article and the relation of our findings to those of previous observers will be discussed.

CONCLUSIONS

1. The secretion of neutral red reproduces those variations which are observed in the mitochondrial apparatus of the renal tubule cells of animals living under native conditions. The tubular absorptive processes concerned with water, salts and sugars do not produce these effects.

2. The changes in the mitochondria consist of both structural and constituent alterations. These have been shown to be not merely phenomena concomitant with secretion, but a determining factor in one part of this process; namely, in the concentration of the dye within the cell.

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EXPLANATION OF PLATES

PLATE 28

Magnification of photographs × 525; of drawings × 680.

FIGS. 1 *a* to 3 *a*, and 1 *b* to 3 *b*, tubules from two animals living under native conditions, Kolster-Altmann procedure. Figs. 1 *a* and 1 *b* show Segment II of the tubule. The cells are filled with long filamentous structures. Figs. 2 *a* and 2 *b* show Segment II from another animal. Its cells are greatly swollen and filled with large round granules. Figs. 3 *a* and 3 *b*, from the same specimen as Nos. 2 *a* and 2 *b*, show sections of Segment IV. The flat cells are filled with heavy rodlets. They contain no granules.

FIGS. 4 *a*, 4 *b* and 5 *a*, 5 *b*. The negative effect of absorptive processes on the filaments of Segment II. Experiment of Table I. Kolster-Altmann procedure. Figs. 4 *a* and 4 *b* show the cells of the kidney removed before the perfusion was started, filled with long filaments and containing no large round granules. Figs. 5 *a* and 5 *b* show the other kidney of the animal after 1¼ hours perfusion with plain Locke's solution. Under these conditions absorption by the tubule cells of water, salts and sugar occurred, but no secretion of any substances. The cells show no alteration from their previous condition. In the right part of Fig. 5 *b* Segment IV is seen with its unaltered rodlets.

PLATE 29

Magnification of photographs × 525; of drawings × 680.

FIGS. 6 *a*, 6 *b*, 7 *a*, 7 *b*, 8 *a* and 8 *b*. The changes produced by the secretion of neutral red on the filaments of Segment II. Experiment of Table II. Kolster-Altmann procedure. Figs. 6 *a* and 6 *b* show the cells of Segment II of the kidney removed after the preliminary perfusion with plain Locke's solution that contained no secretable substances. They are filled with long filaments and contain no granules. Figs. 7 *a* and 7 *b* show Segment II from the other kidney that was perfused with neutral red and which secreted this substance in considerable

amount. The filaments are replaced by large granulovacuoles and no filaments are visible. Figs. 8 *a* and 8 *b* show the unaltered rodlets of Segment IV from the same kidney.

FIGS. 9 *a*, 9 *b*, 10 *a*, 10 *b* and 10 *c*. Effect of the secretion of neutral red on cells of Segment II which originally contained a mixture of filaments and granules. Experiment of page 443. Kolster-Altmann procedure. Figs. 9 *a* and 9 *b* show the condition of the cells of Segment II of the kidney removed before the secretion of neutral red. Many filaments and a certain number of granules are seen. Figs. 10 *a* and 10 *b* show similar cells from the other kidney which had been perfused with and which had secreted neutral red. The filaments have entirely disappeared and the cells are filled with large round granules. Fig. 10 *c* shows the rodlets of Segment IV unchanged.

PLATE 30

All figures, except No. 17, are of fresh preparations of unfixed tissue suspended in Locke's solution.

FIG. 11. Extravital staining with neutral red. Experiment of Table I. A portion of Segment II whose cells have retained their normal position in the tubule. They are filled with granulovacuoles of neutral red. The nuclei are unstained. Magnification × 390.

FIG. 12. Extravital staining with neutral red. An isolated cell from Segment II of the same kidney. Clustered around the nucleus are granulovacuoles of neutral red. Compare the appearance with that of the same tissues after the Kolster-Altmann procedure (Figs. 7*a* and 7*b*) and after Bouin fixation and Gram-Weigert staining (Fig. 23) and Zenker fixation and Gram-Weigert staining (Fig. 24). Magnification × 1200.

FIG. 13. Extravital staining with Janus green. Unfixed cell from Segment II of the kidney of the experiment of Table III. The large granulovacuoles of Janus green are seen as well as the fine bacillary thread-like mitochondria. Note that in this and the following figures mitochondria occur only in parts of the cell free of granulovacuoles. Magnification × 1200.

FIG. 14. Extravital staining with both neutral red and Janus green. Experiment of Table IV. There is an indiscriminate mixing of red and green granules throughout the protoplasm of the cell with a few scattered mitochondria. Magnification × 1200.

FIG. 15. The same specimen and procedure. Another cell from Segment II shows red and green vacuoles as well as some which are stained with a mixture of the two dyes. This mixture is represented for purposes of reproduction as a stipple whereas in fact the admixture of dyes produces an even greenish brown tone. Magnification × 1900.

FIG. 16. The same specimen and procedure. Many fine bacillary forms of mitochondria stained with Janus green are visible. All of the granulovacuolar bodies are stained with neutral red. Magnification × 1200.

FIG. 17. Extravital staining with neutral red, fixation in formol-Zenker's solution and counterstaining by the Gram-Weigert method. Experiment of Table I. A cell from Segment II. Some of the granules have retained their neutral red color, some are heavily stained by the methyl violet, represented as black, while others show a mixture of the two dyes. These latter present an even purplish red tone that has been represented by a coarse stipple. Magnification × 1900.

FIG. 18. Supravital staining with neutral red. A cell from Segment II of the kidney of an untreated animal supravitally stained. The protoplasm is filled with deeply stained red granulovacuoles, and the picture is identical to that obtained by extravital staining. Compare Fig. 12. Magnification × 1200.

FIG. 19. Supravital staining with Janus green. A similar cell from the same kidney illustrated in Fig. 18, supravitally stained. Many green granulovacuolar bodies and a few fine mitochondria are visible. Compare with Fig. 13, an extravitally stained cell. Magnification × 1200.

FIG. 20. Supravital staining with both neutral red and Janus green. Cell of Segment II from same material supravitally stained in Locke's solution containing 1/100,000 neutral red and 1/500,000 Janus green. A mixture of red and green vacuoles is clustered about the nucleus. For similarity to the results of combined extravital staining of the two dyes compare with Fig. 14. Magnification × 1200.

FIG. 21. Supravital staining with neutral red and Janus green. Same material and method. Fine green mitochondria are visible with areas of clustered neutral red granulovacuolar bodies. The corresponding appearance by the combined extravital method is seen in Fig. 16. Magnification × 1200.

FIG. 22. Vital staining with neutral red, with subsequent supravital staining with Janus green. A cell from Segment II of the kidney of an animal vitally stained with neutral red. Material from this kidney was then supravitally stained in Locke's solution containing 1/500,000 Janus green. The vital granulovacuolar bodies of neutral red alternate with areas where the persisting mitochondria have been supravitally stained by the Janus green. The effect is identical with combined extravital (Fig. 16) and combined supravital staining (Fig. 21). Magnification × 1200.

PLATE 31

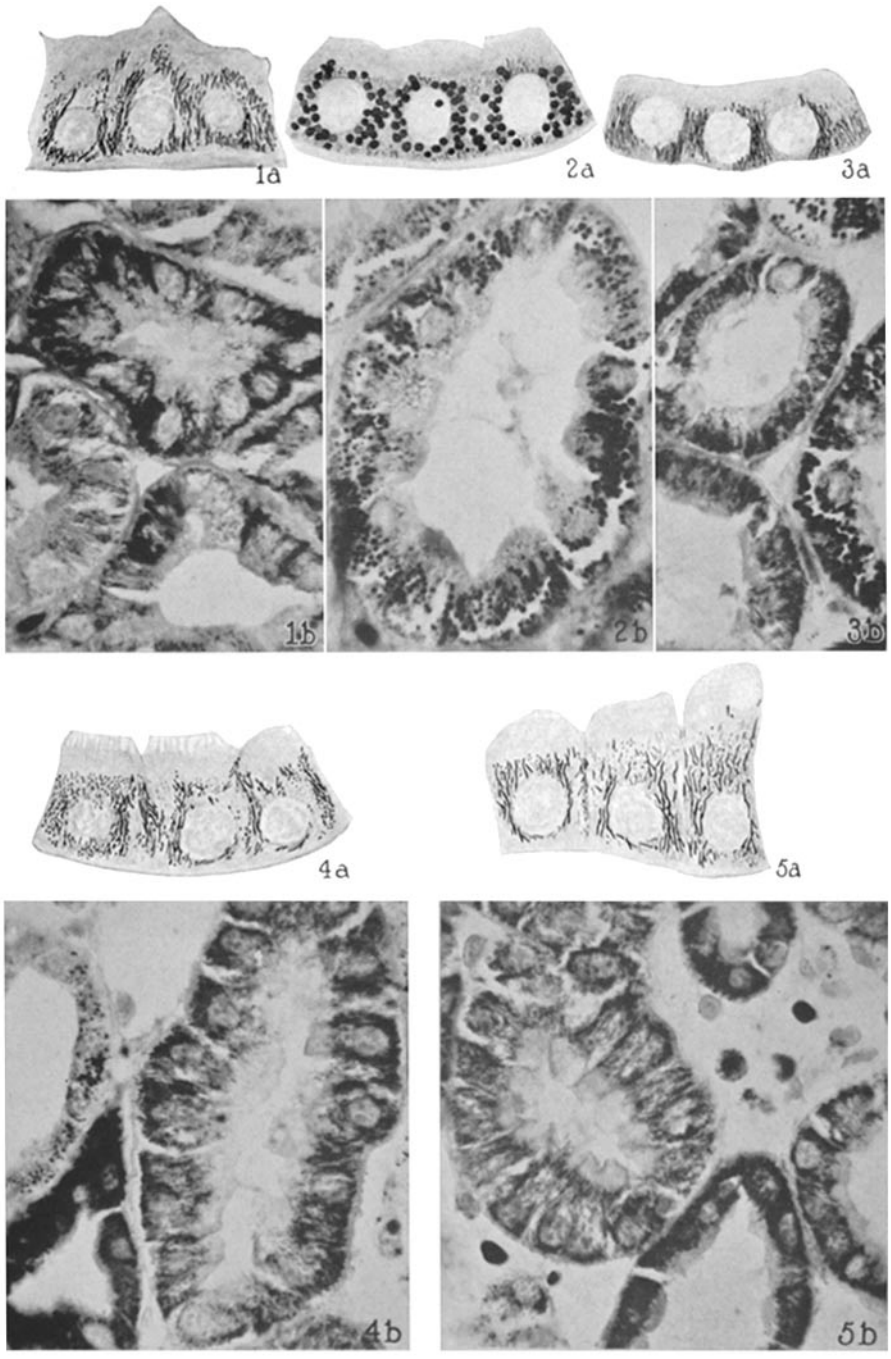
FIG. 23. Segment II of the tubule from the kidney secreting neutral red in experiment of Table II. Bouin fixation, Gram-Weigert stain and no counterstain. The cells are filled with granulovacuoles that have resisted the action of acetic acid and retained methyl violet. Compare with Figs. 7 *a* and 7 *b* where the same structures are stained by the Kolster-Altmann procedure. Magnification × 525.

FIG. 24. From the same kidney. Zenker fixation, Gram-Weigert stain. The same Gram-positive granules fill the cells. Magnification × 525.

FIG. 25. Segment II of a normal animal living under native conditions, Kolster fixation, Gram-Weigert stain. The cells are filled with Gram-positive granulo-

vacuoles. The same specimen with the Altmann stain is shown in Figs. 2 *a*, 2 *b*. Magnification × 525.

FIG. 26. Segment II of another normal untreated animal. Kolster fixation, Gram-Weigert stain. No Gram-positive granules are visible, as the cells in this instance are filled with mitochondrial filaments. Compare Figs. 1 *a* and 1 *b*, a section from the same block stained by the Altmann method. Magnification × 525.



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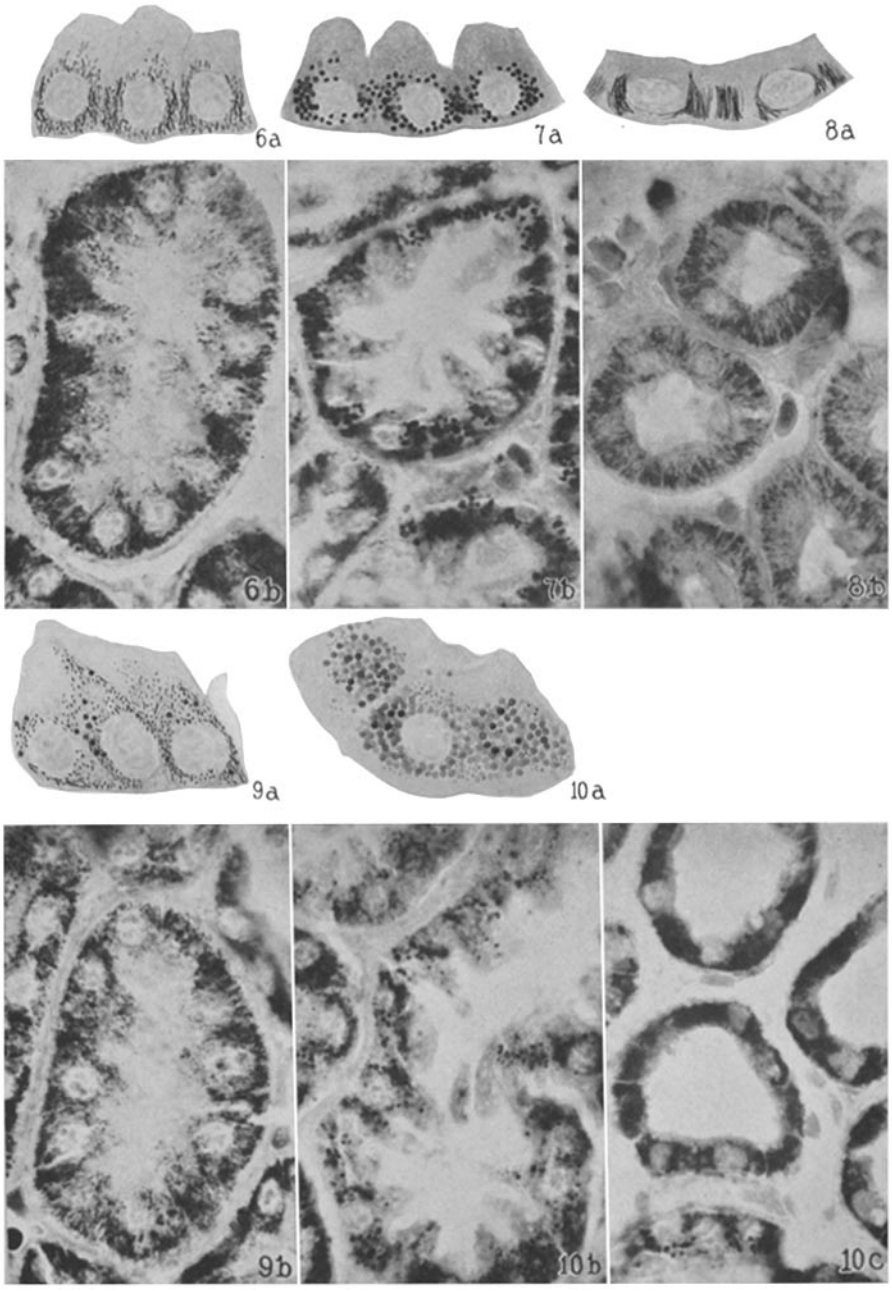

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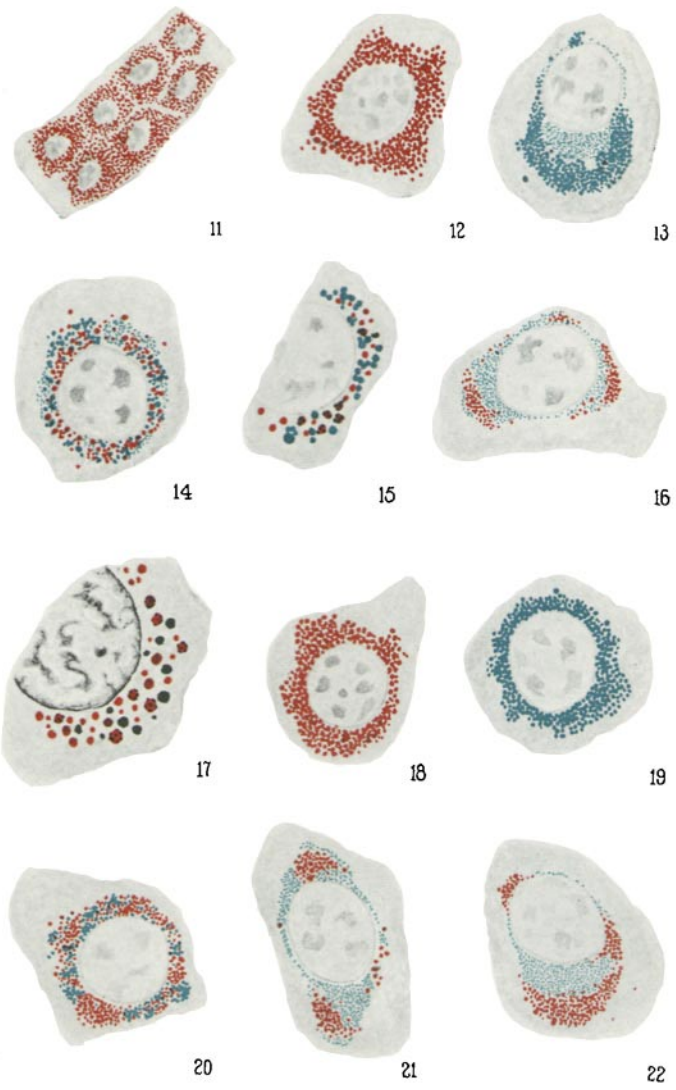

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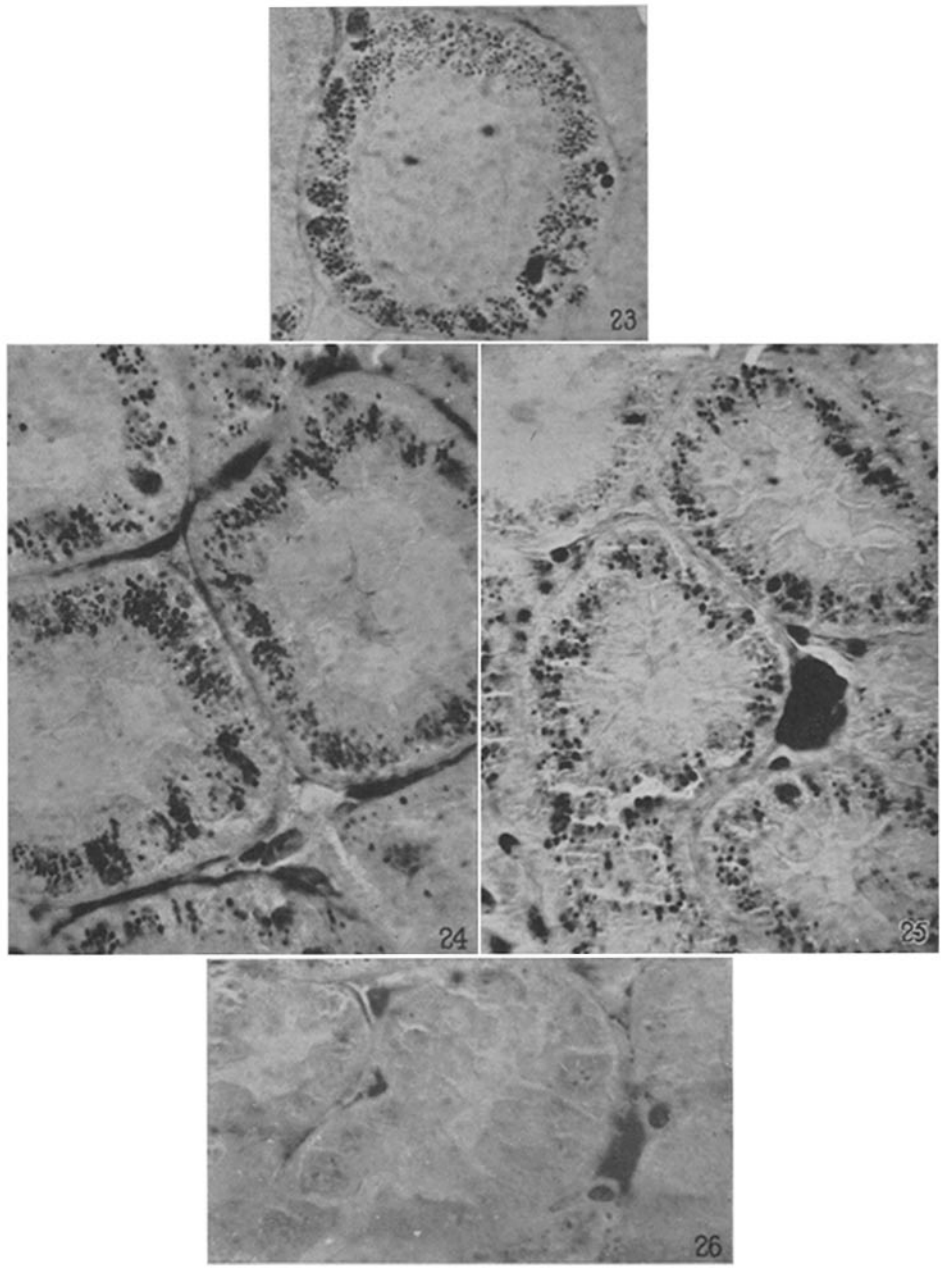

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