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

## II. The Functional Phase of the Secretory Mechanism*

By JEAN R. OLIVER, M.D., and EDNA MORRIS LUND

*(From the Department of Pathology of the Long Island College of Medicine, The Hoagland Laboratory, Brooklyn)*

Plate 32

(Received for publication, October 24, 1932)

Although the morphological aspects of the secretory process have allowed us to form some idea as to what occurs when the dye enters the cell body from the blood stream and have shown us the mechanism by which it is concentrated there, nothing has been learned from such data as to the final definitive act of secretion; that is, the discharge of the dye into the tubule lumen. The dye was left in our description concentrated in vacuoles. But these vacuoles neither moved through the cell nor did they burst and liberate their content into the lumen nor were they discharged into it as such. It is true that such mechanical methods of renal cell discharge have been described by isolated investigators but the great majority of workers have denied their occurrence. Certainly the newer methods we have used would have disclosed such gross phenomena if they had been present. *A priori* it is therefore certain that since the discharge of the dye is not associated with structural change its mechanisms must be investigated by other than morphological methods. And here another advantage of the extravital method, combining as it does in the same experiment the two aspects of cell activity, is evident, for the same experiments that we have used can be also employed in obtaining evidence concerning the less obvious phenomena of the final act of secretion.

Since we have already examined the morphological evidence of

* This investigation has been made with the assistance of a grant from the Josiah Macy, Jr., Foundation.

concentration in the cell we shall begin with this feature of the secretory process.

### *The Determination of the Concentration of Neutral Red in the Tissues of the Secreting Kidney*

Experiments were performed in the manner described in the foregoing paper. Neutral red was perfused through the venous circulation of the tubules while clear Locke's passed through the glomerular circulation. The urine was collected as usual in 15 minute periods and the dye content determined. After from 3 to 5 hours, the experiment was stopped. The kidneys were then removed intact, freed of all adventitious tissues, dried by repeated blotting until no more

TABLE I

*Concentration of Neutral Red in the Kidney Tissue from a Solution of Locke's Containing 1.25 Mg. per 100 Cc.*

| Average rate of secretion of neutral red over long period experiment | Neutral red in kidney tissue | Concentration factor $\frac{\text{Kidney tissue}}{\text{Perfusion fluid}}$ |
|---|---|---|
| *mg. per hr.* | *mg. per 100 gm.* | |
| 0.03 | 411 | 328 |
| 0.03 | 354 | 283 |
| 0.09 | 782 | 626 |
| 0.10 | 939 | 751 |
| 0.25 | 2250 | 1800 |
| 0.30 | 420 | 336 |
| 0.50 | 261 | 208 |
| 0.60 | 419 | 335 |
| 0.80 | 446 | 332 |

fluid came from their dull surfaces and weighed. Each was then ground with weak acid alcohol in a mortar until only a few bits of colorless fibrous tissue remained. After centrifugalization the dye content in mg. per 100 gm. of kidney substance was determined colorimetrically and the concentration in the kidney tissue as compared to that of the perfusion fluid calculated. Table I shows some of the results. It will be seen that there were considerable variations in the concentration of dye in the tissues. These may be explained by the varying length of time of the perfusion and the efficacy of it which depends in turn on the flow through the vessels. This flow varies considerably due to irregularities in vascular tone. We need not, however, consider the cause of variations in the concentrating process here but shall confine ourselves to points that immediately concern the problem of the output of the dye. That the concentration in the cells is not directly concerned with this essential phase of secretion is shown by the fact that

the output of dye bears no direct relation to the concentration within the cell. As much or more dye was secreted per hour with a concentration in the cell body 336 times that of the perfusion fluid as when the intracellular concentration was 1800 times. And of the same significance is the observation that with a gradually increasing concentration of dye in the tissues, a fact that may be roughly determined during the course of the experiment by gross examination of the kidney's color, there may go a decreasing rate of elimination into the urine.

The result of these findings indicates at once that the older conception of secretion whereby concentration in the cell body was assumed to be the preliminary step and determining factor in the passage of the dye into the lumen of the tubule is far too simple. Output and intracellular concentration must be considered separately. Our first step was to determine how great a part the concentrating process in the granulovacuolar structures may play in the output of dye into the urine.

### *The Contribution of the Concentrating Granulovacuolar Structures to the Output of Dye*

In the perfused organ it is possible at the height of the secretion of the dye to suddenly change the dye-containing fluid that is coming by way of the venous system to the tubules so that the cells are at once bathed with clear Locke's which contains no dye. It is evident that under such altered conditions any dye that is secreted after the change has been made, must of necessity be derived from that which has been concentrated in the cell. This amount compared to that which was being eliminated when the cells were receiving dye from fluid in the vessels in the period before the change, will allow us to estimate what part may be contributed by the concentrating process to secretion. The experiment was performed as follows:

The kidneys were perfused in the usual manner, neutral red passing only to the tubules. After three periods of fairly constant secretion of the dye the bottle containing neutral red that was supplying the tubules was replaced by one containing the same clear Locke's as was passing to the glomeruli. The connecting tubing and cannulae were flushed free of the dye-containing Locke's so that in the course of 1 minute or less a change had been made and the tubules were receiving no dye whatsoever. The collections of urine were continued for five or six periods. The details of a typical experiment are shown in Table II and the results of several are illustrated in Text-fig. 1. In this chart the amount of neutral red excreted

in the last period when the cells were obtaining dye from the vessels is taken as 100 per cent and the amount excreted in the latter periods when the cells were receiving no dye is expressed as a percentage of this amount. It will be seen that there was a rapid and progressive fall in the amount excreted which, after three or four periods, reached a fairly constant level at about 5 per cent of the previous secretion. The first sample studied, collected 15 minutes after the change, cannot be considered to indicate the amount of dye obtained from the concentrating vacuoles alone, for immediately after the change to clear fluid was made there must have been a certain amount of dye free within the cells in the process of elimination and this was obtained in the first and perhaps the second sample collected. The importance of this fact will be referred to later.

TABLE II

*The Contribution to the Urine of the Dye Concentrated in the Kidneys*

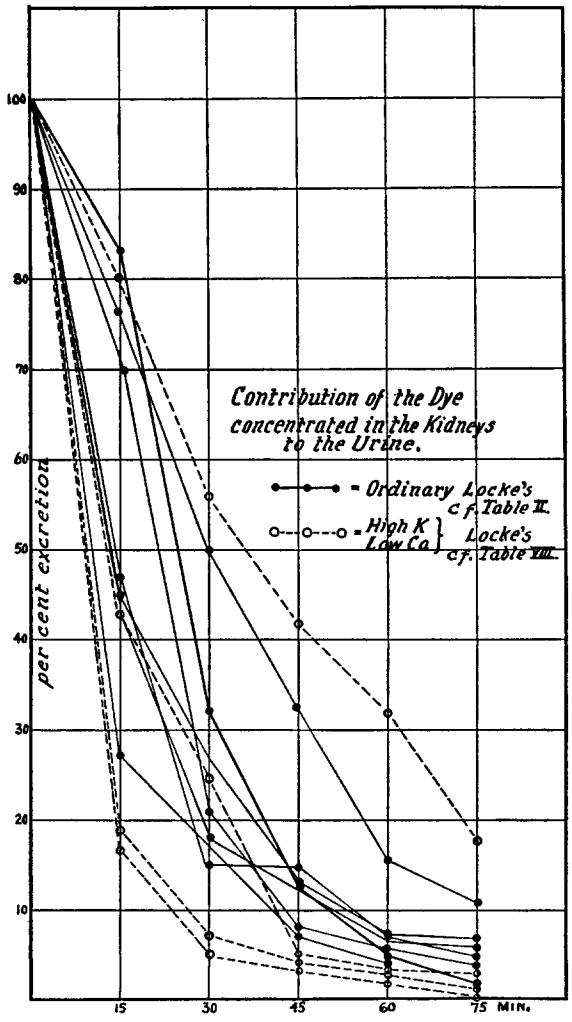
| Time | Arterial flow | Venous flow | Urine volume | Dye | Salt in perfusion fluid | Sugar |
|---|---|---|---|---|---|---|
| | *cc. per hr.* | *cc. per hr.* | *cc. per hr.* | *mg. per hr.* | *per cent* | |
| | Tubules perfused with 1.25 mg. per 100 cc. Locke's solution | | | | | |
| 11:00–11:15 | 320 | 400 | 5.2 | 0.34 | 40 | 0 |
| 11:15–11:30 | 320 | 400 | 6.0 | 0.32 | 40 | 0 |
| 11:30–11:45 | 320 | 320 | 7.2 | 0.20 | 40 | 0 |
| | Clear Locke's to tubules of kidneys | | | | | |
| 11:45–12:00 | 320 | 320 | 6.0 | 0.08 | — | 0 |
| 12:00–12:15 | 320 | 400 | 5.2 | 0.04 | 45 | — |
| 12:15–12:30 | 240 | 320 | 4.4 | 0.02 | — | 0 |
| 12:30–12:45 | 240 | 320 | 4.0 | 0.01 | — | — |
| 12:45–1:00 | 240 | 400 | 4.0 | 0.01 | 45 | 0 |

Concentration of dye in kidneys = 424 mg. per 100 gm. = 336 times concentration in original perfusion fluid.

A confirmation of the small part played by the dye concentrated within the vacuoles in the actual output of dye into the lumen can be obtained in another way as the following experiment shows.

The kidneys of an animal were perfused as described above with neutral red Locke's solution and the urine collected, the samples from each kidney being kept separate. After three 15 minute periods had passed, the left kidney was removed with ligation of all the cut vessels and without disturbing the right kidney. The bottle of dye-containing Locke's that was now perfusing only the right kidney was replaced with clear Locke's and the collections continued from this one kidney until a minimum amount of dye was excreted. The right kidney was now removed and the concentration of dye in the two organs determined. A comparison shows that

though there is less dye in the right kidney than in the left, 622 mg. per 100 gm. as against 904 mg. per 100 gm., this disproportion is insignificant as compared to that




TEXT-FIG. 1

existing between the amount of the dye that the two kidneys were secreting at the moment of their removal; namely, 0.005 mg. per hour and 0.100 mg. per hour.

Another phenomenon seen in this experiment should be noted although it does not concern the secretory process. The greater part of the dye that was removed

from the kidneys in these experiments did not pass into the urine but back into the Locke's solution in the blood vessels. As this fluid was collected in the outflow bottle from the vena cava after passing through the kidneys it was definitely stained with dye. That such a back flow from kidney tissue to blood vessels may occur in the living animal has been suspected and, in the case of urea, actually demonstrated (1).

The conclusion to be drawn from these experiments is that only a small amount, probably $\frac{1}{10}$ or $\frac{1}{20}$, of the dye that appears in the urine at the height of secretion under ordinary conditions can be derived from the granulovacuolar mechanisms.[1] The bulk of the dye present in the urine must have entered the cells from the blood vessels and passed through them to be discharged directly into the lumen of the tubules independently of these structures. The question may therefore be asked as to how much of the dye that enters the cell is concentrated in the granulovacuolar apparatus and thus withheld for a certain time from elimination and how much is directly discharged into the lumen of the tubule.

### *A Factor That Determines the Processes of Concentration and of Elimination*

It can be shown that one factor which influences the distribution of the dye between concentration in the vacuoles and direct elimination into the lumen is the concentration of it in the blood stream. The following experiment illustrates this point.

The kidneys of an animal were perfused through the venous system with Locke's solution containing a low concentration of neutral red of 0.3 mg. per 100 cc. The procedure was identical with that of the experiment illustrated in Table II. Table III shows the result of a series of such experiments. The next group of experiments was done in exactly the same way except that the dye in the solution going

[1] The objection may be raised that under the conditions of the experiments just described the failure of the dye to pass from the vacuoles into the urine is due to a lack of passage of it into the vacuoles from the blood stream; that a continuous intake into the vacuole is necessary for its output. But our first experiments have shown that, with a continuous supply and even with an increasing concentration in the vacuoles, elimination nevertheless falls, a fact to be emphasized by other experiments that follow (Table V). So too it will be shown that the passage of the dye from a concentrated phase to one less concentrated is not dependent on any such continuing supply of dye to the concentrated phase (page 475).

to the tubules was in the higher concentration of 1.25 mg. per 100 cc. of Locke's solution. The findings are also shown in Table III.

If the ratio of dye eliminated to the concentration of it in the vessels is compared in the two cases it is seen that a higher ratio of elimination is found with a high blood concentration than with a low one. On the other hand, the relative degree of concentration in the kidney is higher when the concentration in the vessels is low. This allows us to conclude that the concentration in the blood is one factor that determines how much of the entering dye is concentrated in the granulo-

TABLE III

*Effect of Concentration of Dye in Perfusion Fluid on Various Secretory Processes*

| Average optimal direct secretion* of neutral red | Ratio: Rate of secretion / Concentration in perfusion fluid | Concentration factor in kidney tissue |
|---|---|---|
| Perfusion fluid = 0.3 mg. neutral red per 100 cc. | | |
| 0.018 | 0.06 | 1005 |
| 0.023 | 0.07 | 1408 |
| 0.030 | 0.10 | 1036 |
| 0.019 | 0.06 | 1561 |
| Perfusion fluid = 1.25 mg. neutral red per 100 cc. | | |
| 0.60 | 0.48 | 335 |
| 0.80 | 0.64 | 332 |
| 1.1 | 0.88 | 208 |

| | Low concentration of neutral red | High concentration of neutral red |
|---|---|---|
| Relative values of concentration of dye in perfusion fluid... | 1 | 4+ |
| Average relative values of ratios of direct secretion..... | 1 | 9+ |
| Average relative values of concentration factor in tissues.... | 3+ | 1 |

* A definition of the term direct secretion is given on page 467.

vacuolar bodies and how much is directly and immediately eliminated. Another factor which doubtlessly affects the amount of dye stored in the vacuoles is the degree of their saturation with the dye. Unfortunately we have been unable to investigate this point for reasons that will appear later.

### *The Demonstration of Similar Concentrating Processes during the Secretion of Neutral Red by the Living Animal*

Though our previous experience with the extravital method has led us to believe that the activities occurring under its conditions are similar to those occurring in the living animal, it seems best to search

under vital conditions for processes analogous to those just described. It is common knowledge that neutral red appears in the urine shortly after its injection into the blood stream of an animal and that it is concentrated in the kidney epithelium for some time, conditions which are essentially those described above as occurring in extravital kidneys. We have tried, however, to obtain some quantitative data in regard to the relations involved in the living animal.

A series of frogs were stained vitally by the repeated injection of 0.25 per cent solution of neutral red into their dorsal lymph sacs. After three injections the animals were anesthetized with urethane and a portion of the left kidney removed. All bleeding was stopped by ligature, the wound was closed by sutures and the animals replaced in their tank. They recovered from the anesthetic and seemed entirely normal. The concentration of neutral red in the portion of kidney removed was now determined as previously described. 20 hours later the animals were killed and the dye content remaining in their kidneys determined. The results are shown in Table IV. Although there is considerable variation it will be noted that after 20 hours the slow elimination of the dye which had been concentrated in the kidney was continuing.

TABLE IV

*Concentration of Dye in Kidneys of Living Animal*

| Original dye in kidney | Dye in kidney after 20 hrs. | Per cent remaining |
|---|---|---|
| *mg. per 100 gm.* | *mg. per 100 gm.* | |
| 43.8 | 2.4 | 5.4 |
| 36.1 | 5.6 | 15.5 |
| 30.0 | 5.6 | 18.0 |
| 25.7 | 1.8 | 7.0 |
| 16.6 | 7.2 | 43.0 |

It is apparent from the results of the experiments done so far that the secretion of neutral red by the renal cells is not a single unified procedure but that its elimination must be divided into two very different processes which are independent to a large degree of each other. The first type of secretory process occurs only when there is a considerable concentration of the dye in the blood vessel, it occurs promptly and is of considerable amount. The second is a slow long continued elimination which may proceed after the dye has completely

disappeared from the blood stream and this elimination is always low in its rate. The mitochondrial granulovacuolar apparatus of the renal cell plays no observable part in the first type of secretion, but is definitely the controlling mechanism of the second. Since throughout the remainder of our discussion we must distinguish between these two processes that, combined, result in what has been called "secretion," we shall call the first, direct, and the second indirect secretion. The latter term implies the fact that the dye which ultimately reaches the lumen of the tubule has passed indirectly to it by the intermediate vacuoles.

TABLE V

*Fall in Direct Secretion of Neutral Red*

| 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* | |
| Tubules perfused with 1.25 mg. neutral red per 100 cc. Locke's solution | | | | | | |
| 10:00–10:15 | 440 | 920 | 12 | 0.78 | 45 | 0 |
| 10:15–10:30 | 440 | 800 | 11 | 0.89 | — | 0 |
| 10:30–10:45 | 520 | 920 | 11 | 0.92 | — | — |
| 10:45–11:00 | 440 | 800 | 10 | 0.73 | 40 | — |
| 11:00–11:15 | 440 | 920 | 10 | 0.52 | — | 0 |
| 11:15–11:30 | 360 | 800 | 8 | 0.39 | — | — |
| 11:30–11:45 | 440 | 920 | 10 | 0.31 | 45 | 0 |
| 11:45–12:00 | 440 | 920 | 10 | 0.30 | 45 | 0 |

The mechanism by which indirect secretion operates, the mitochondrial granular apparatus, is known and, since this seems to play no part in direct secretion, our next problem is a search for the controlling mechanisms of this process. One thinks at once of membranes and changes in their permeability as controlling factors in the passage of the dye. An attempt, therefore, was made to see if such a concept may be applied to explain the variations occurring in the direct secretory process. The following experiment shows that local cellular changes must influence the output of the dye into the lumen and since these variations are rapid in their development and of considerable degree it is certain that they must be affecting the direct and not the indirect secretion of the dye.

The kidneys of an animal were perfused in the usual manner, the tubules receiving neutral red in the usual concentration of 1.25 mg. per 100 cc. Locke's solution.

Table V shows the flow through the vessels of the two systems and the elimination of water, salts and dye. It will be observed that there was a marked decrease in the secretion of the latter though the rate of salt and water secretion remained approximately constant.[2] In this experiment the cause of the decrease in the secretion must be a local cellular one for the rate of flow through the blood vessels did not significantly vary and the concentration of dye in them was constant. The spinal cord had been destroyed so that nervous effects were at a minimum.

Is this local effect on some specific phase of the direct passage of the dye through the cells into the lumen and if so can we more accurately locate the site of the changes that produce it? The next experiment answers this question.

TABLE VI

*Effect of Bichromate on Direct Secretion*

| 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* | |
| 10:15–10:30 | 520 | 800 | 6.4 | – | 35 | 0 |
| 10:30–10:35 | 15 cc. potassium bichromate to tubules | | | | | |
| 10:45–11:00 | 520 | 600 | 5.7 | – | 41 | Tr. |
| 11:00–11:15 | 560 | 800 | 9.6 | – | 50 | + |
| | 1.25 mg. neutral red in 100 cc. Locke's solution to tubules | | | | | |
| 11:15–11:30 | 560 | 800 | 12.0 | 0.039 | 52 | + |
| 11:30–11:45 | 520 | 800 | 10.4 | 0.040 | 50 | + |
| 11:45–12:00 | 520 | 800 | 10.4 | 0.030 | 52 | + |

Concentration of dye in kidneys 782 mg. per 100 gm. = 626 times concentration in perfusion fluid.

## *A Specific Alteration in Direct Secretion as a Result of Inner Membrane Damage without Impairment of the Concentrating Processes of Indirect Secretion*

The kidneys were perfused as usual with clear Locke's solution passing to both the tubules and the glomeruli. After urine formation was normally established,

[2] Such a gradual fall in the elimination of neutral red is almost constantly observed in any perfusion of the isolated kidney. It explains, for example, the rather marked differences in the rate of elimination of the dye given in Tables III and I. In the former the figures represent the average of the optimal direct secretion as found in the first periods of a perfusion, the latter the average of the rates of elimination in longer experiments in which direct secretion had to a greater or less degree fallen off.

15 cc. of potassium bichromate in Locke's solution in a dilution of 1/10,000 was passed to the tubules through the cannulae supplying the venous system. The effect on the formation of the urine is shown in Table VI. The failure of absorptive processes is evident in the increase in water and salt and in the appearance of sugar (12). Neutral red in the usual concentration of 1.25 mg. per 100 cc. Locke's solution was now passed to the tubules and the collection of urine continued. It is observed that only very small amounts of dye were eliminated, only a faint pink grossly visible with the naked eye as contrasted to the Burgundy-red urine that appears in the first periods of an experiment from a normal kidney (compare Table V). At the close of the experiment the kidneys were removed and their dye content examined by the method previously described. A concentration 626 times that of the perfusion fluid was found, a figure well within the range of the dye content previously found in normally secreting kidneys (compare Table I).

A consideration of these results shows definitely that the action of the bichromate was not in preventing the dye entering the cell nor in any change in the cell's ability to hold it concentrated in the vacuolar apparatus. And since it did not reach the lumen in normal amount the change producing the decreased elimination must therefore have occurred at the inner cell surface adjoining the tubule lumen.[3]

That the effect in the above experiment was limited at least relatively to the inner (lumen) membrane has been easily demonstrated. A question somewhat more difficult to answer arises when one asks if the outer (vascular) membrane may be in turn specifically altered. The observations of Sato (3) and of Gellhorn (4) on the effect of ions on the permeability of cell membrane are suggestive. But before speculating on the possibility of ion action and its exact situation we must first demonstrate that such action affects the passage of the dye through the cells of the secreting kidney.

### *The Increased Passage of Dye through the Renal Cells as a Result of Ion Action*

The possible action of an alteration of the ionic balance in the Locke's solution on the passage of dye through the cells may be particularly well examined in kidneys in which the not uncommon fall in dye elimi-

[3] We are not here concerned with the mechanisms of the absorptive processes in the cell but the failure of absorption of water, salt and sugar is presumptive evidence that the permeability of the inner membrane is also decreased in an opposite direction to that of the secretory process.

nation from its original figure has occurred during the course of a normal perfusion experiment. The findings of Gellhorn (4) and Sato (3) suggest that this decrease in passage of the dye might possibly be overcome by increasing the concentration of K ions in the Locke's solution or by decreasing the Ca ions. The following experiment examines this point.

The kidneys were perfused in the usual way, with ordinary Locke's solution containing neutral red in a concentration of 1.25 mg. in 100 cc. passing to the tubules

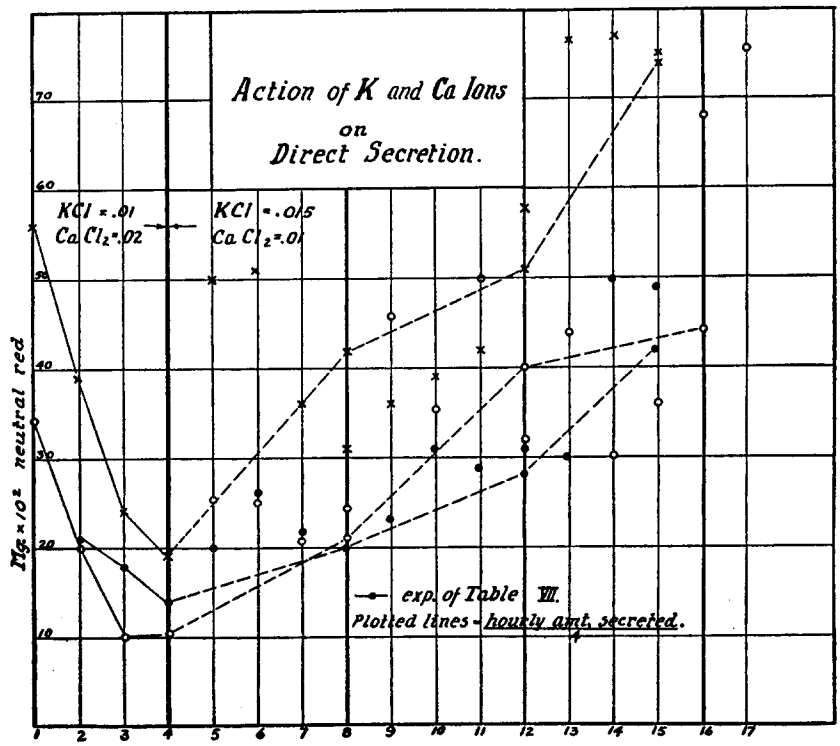
TABLE VII

*Effect of K and Ca Ions on Direct Secretion*

| 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* | |
| 1.25 mg. of neutral red in 100 cc. Locke's solution to tubules, KCl = 0.01 per cent, $CaCl_2$ = 0.02 per cent | | | | | | |
| 10:10–10:15 | 400 | 840 | 7.0 | 0.21 | 40 | 0 |
| 10:15–10:30 | 420 | 840 | 6.0 | 0.18 | 40 | 0 |
| 10:30–10:45 | 440 | 840 | 6.8 | 0.14 | 40 | 0 |
| Neutral red as before, KCl = 0.015 per cent, $CaCl_2$ = 0.01 per cent | | | | | | |
| 10:45–11:00 | 400 | 800 | 7.2 | 0.20 | 40 | 0 |
| 11:00–11:15 | 440 | 840 | 8.0 | 0.26 | 45 | 0 |
| 11:15–11:30 | 400 | 840 | 7.2 | 0.21 | 40 | 0 |
| 11:30–11:45 | 400 | 840 | 6.2 | 0.19 | — | — |
| 11:45–12:00 | 400 | 800 | 6.0 | 0.23 | — | — |
| 12:00–12:15 | 400 | 800 | 5.7 | 0.31 | 45 | 0 |
| 12:15–12:30 | 440 | 840 | 6.0 | 0.29 | — | — |
| 12:30–12:45 | 400 | 840 | 6.0 | 0.31 | — | — |
| 12:45–1:00 | 360 | 800 | 6.0 | 0.30 | 40 | 0 |
| 1:00–1:15 | 320 | 800 | 4.0 | 0.50 | — | 0 |
| 1:15–1:30 | 320 | 800 | 4.0 | 0.49 | 45 | Tr. |

and clear Locke's to the glomeruli. In such a solution the KCl concentration is 0.01 per cent and the $CaCl_2$ concentration 0.02 per cent. Table VII shows the results. It will be seen that as the perfusion continued there occurred a gradual and progressive fall in the rate of elimination of neutral red from 0.21 to 0.14 mg. per hour. When the rate of secretion of the dye had reached this last figure the fluid in the bottle supplying the tubules was changed to a Locke solution containing an increased concentration of KCl, 0.015 per cent, and a decreased concentration of $CaCl_2$, 0.01 per cent. The fluid contained the same amount of neutral red as previously and its pH was not significantly altered by the changes in salt concentra-

tion. It will be seen in Text-fig. 2 that there occurred a gradual progressive increase in the elimination of the dye which ultimately more than doubled the original output of the kidney. It is, moreover, noteworthy that this increase was a long continued phenomena, lasting over a period of 2¾ hours, a striking fact when one considers the marked depressive effect of time in the first part of the experiment. Two other experiments of similar nature are also shown in Text-fig. 2.




TEXT-FIG. 2

Such findings leave no doubt that an increase in the KCl/$CaCl_2$ ratio increases the rate of elimination of neutral red, but this conclusion strictly examined can state no more than that an increased amount of dye entered the tubule lumen under such perfusion conditions. There are at least three ways in which such a result might come about: the inner (lumen) membrane might have become more permeable and thus allowed dye within the cell to enter the lumen more readily; the

dye concentrated within the vacuoles might have been liberated and thus have become available for elimination; or there may have occurred an increased permeability in the outer (vascular) membrane of the cell with a resulting entrance of more dye and hence greater passage of it through the cell. It is not necessary to discuss the probability of these possibilities, for the seat and method of action can be directly demonstrated by experimental means.

TABLE VIII

*Lack of Effect of K and Ca Ions on Inner (Lumen) Membrane*

| 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* | |
| Tubules perfused with 1.25 mg. neutral red per 100 cc. Locke's solution, KCl concentration = 0.01 per cent, $CaCl_2$ concentration = 0.02 per cent | | | | | | |
| 10:15–10:30 | 500 | 600 | 9.0 | 1.3 | 40 | 0 |
| 10:30–10:45 | 400 | 500 | 6.0 | 1.0 | 40 | 0 |
| 10:45–11:00 | 400 | 640 | 8.0 | 1.6 | — | 0 |
| 11:00–11:15 | 440 | 640 | 8.0 | 1.3 | 45 | 0 |
| Clear Locke's to tubules, KCl concentration = 0.015 per cent, $CaCl_2$ concentration = 0.01 per cent | | | | | | |
| 11:15–11:30 | 400 | 600 | 7.6 | 0.56 | 45 | 0 |
| 11:30–11:45 | 400 | 600 | 7.6 | 0.07 | — | — |
| 11:45–12:00 | 400 | 640 | 6.4 | 0.05 | 45 | 0 |
| 12:00–12:15 | 360 | 640 | 6.5 | 0.01 | — | — |
| 12:15–12:30 | 360 | 640 | 5.8 | 0.01 | 45 | 0 |

### *The Site of Ion Action on the Cell Membranes*

To examine this question the method of experiment previously described was used in which the intake of dye into the cells is suddenly stopped in the course of active secretion. In the present experiment, however, the perfusion after this stoppage was continued not with the ordinary Locke's but with this fluid so modified as to contain a high ratio of $KCl/CaCl_2$. The results of such procedure were then compared to the earlier experiments with ordinary Locke's solution. A typical experiment was done as follows. Its results are shown in Table VIII.

The tubules of the kidney were perfused with ordinary Locke's solution containing the usual amounts of KCl in 0.01 per cent, $CaCl_2$ in 0.02 per cent and

neutral red in 1.25 mg. per 100 cc. After four periods the elimination of dye was 1.3 mg. per hour and the kidneys were stained a deep mahogany-red from the dye concentrated within them. At this point the bottle supplying the tubule was changed for one of dye-free Locke's solution containing a high KCl concentration of 0.015 per cent and a low $CaCl_2$ concentration of 0.01 per cent. The perfusion was continued for five periods and the output of neutral red determined. It is seen that a marked drop in elimination was noted in the first period following the change to clear Locke's, as great a one, in fact, as was observed in the previous experiments where the dye-free Locke's solution contained the usual amounts of KCl and $CaCl_2$. Several such experiments are shown in comparison with these earlier ones in Text-fig. 1.

The conclusion is definite from these findings that no more dye is liberated from the cells by the Locke's solution containing a high ratio of $KCl/CaCl_2$ than is freed by that containing the usual ratio. And this fact definitely precludes the possibility that the increased elimination noted in our previous experiments with high ratio Locke's solution could have been the result either of a liberation of the dye in the vacuolar apparatus of the cell or of an increased permeability of the inner (lumen) membrane. If the latter had occurred even without the former there must have been at least a temporary increase in the dye output in the first period following the change to the dye-free perfusion fluid, for the cells contained, as a result of their previous perfusion with dye solution, a certain amount of this material available for secretion. It was therefore only the direct secretory activity of the cell that was affected by the high ratio of KCl to $CaCl_2$ and the exact point of the effect of the ions must have been on the external (vascular) membrane. The effect was to increase its permeability.

These experiments allow us to draw an even sharper differentiation between the indirect secretion of neutral red with its controlling mechanism, the granulovacuolar apparatus, and the direct method of secretion, by their demonstration that this latter process has an entirely different mechanism from the former. This mechanism depends on membranes, and so delicate is its balance that it has been possible by means of special methods to not only dissociate its action from that which controls indirect secretion but to recognize disturbances in either one of the two membranes, inner and outer, that are concerned in the process of passage of dye through the cell.

It is easy to understand, in view of the relative complexity of these

membrane changes compared with the single process of concentration in the vacuolar structures, why the course of the direct secretion of the dye varies so markedly as compared to the relative stability in the operation of the indirect secretory process.

The extreme stability of the indirect method of secretion under adverse conditions is a remarkable fact. Kidneys that are being perfused with a solution of neutral red, though they not infrequently fail to eliminate neutral red in a normal manner, never fail to take up and concentrate the dye in their granulovacuolar system and to proceed with its slow indirect secretion. Why is the latter so markedly resistant to the effect of damage?

### *The Degree of Stability of the Concentrating Processes of Indirect Secretion*

A reason for this marked resistance is shown in the following experiment.

Kidneys were perfused in the usual manner with clear Locke's solution passing to both glomeruli and tubules. Table IX shows the results. After normal function was established, 20 cc. of 1/1000 corrosive sublimate in Locke's solution was passed by way of the veins to the tubules. Following this, neutral red in a concentration of 1.25 mg. in 100 cc. was added to the clear Locke's solution supplying the tubules. As will be seen in the table very little dye reached the urine which was scanty in amount, high in salt and contained sugar. The perfusion was continued for six periods and the kidneys then removed. Their appearance was strikingly different from that of normally stained kidneys or from those which had been stained after small doses of potassium bichromate, for they were now a brick-reddish yellow in color and very firm in consistency. Fresh crushed specimens showed no evidence of vacuoles or any other granular or filamentous structures in the cells. The protoplasm was stained a definite yellowish hue instead of the mahogany-red seen in the normal kidneys and even the nucleus was tinged by the yellow color. The appearance of these obviously dead cells in fixed and stained sections was similar to that previously described (5) as a result of sublimate under extravital conditions (Fig. 1).

The appearance of the fresh tissue showed a definite shift towards the alkaline side in the reaction of the dye within the cell but it did not appear from either gross or microscopical examination that there had been any definite concentration of the dye above that which existed in the perfusion fluid. However, when the kidneys were weighed and the dye extracted, it was found that it was 18 times as concentrated in the dead kidney tissue as in the fluid which had bathed the cells.

It is plain from these experiments that even in dead kidney cells there still occurs a concentration of neutral red above that of the perfusion fluid and that the process of concentration therefore does not depend on any vital activity of the cells but on the nature of the constituents of their protoplasm. It is possible in fact to reproduce to a certain extent all the phenomena of the indirect secretory process with a physical model.

TABLE IX

*Effect of Corrosive Sublimate on Concentration of Dye in Kidney*

| 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* | |
| Perfusion of tubules with clear Locke's solution | | | | | |
| 600 | 800 | 5.6 | – | 40 | 0 |
| 600 | 800 | 6.0 | – | 45 | 0 |
| 20 cc. 1/1000 sublimate to tubules | | | | | |
| 600 | 600 | 7.2 | – | 70 | + |
| Neutral red 1.25 mg. per 100 cc. Locke's solution to tubules | | | | | |
| 600 | 640 | 3.0 | – | – | – |
| 640 | 800 | 2.4 | 0.015 | 71 | + |
| 640 | 640 | 1.4 | 0.008 | 71 | + |
| 700 | 800 | 1.2 | – | – | – |
| 640 | 800 | 1.2 | – | – | – |
| 600 | 640 | 1.2 | 0.007 | 75 | + |

Concentration of dye in kidneys = 22.6 mg. per 100 gm. = 18 times concentration in perfusion fluid.

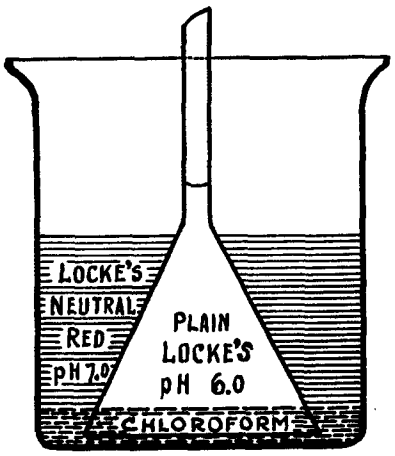
## *The Resemblance between a Physical Model and the Indirect Secretory Processes*

The model used in this demonstration is that devised by Irwin (6) and Osterhout (7) for the demonstration of the manner of entrance of dyes into living cells.

Text-fig. 3 shows the arrangement of the solutions which consisted of Locke's solution containing neutral red in the concentration used in the perfusion fluid at a pH of 7.0 as the outer fluid, chloroform, which represents the concentrating granulo-vacuoles of the cell, and the inner fluid, representing the urine in the tubules, which consisted of plain Locke's solution at a pH of 6.0. All three phases of the model were repeatedly stirred and the fluid in the inner chamber kept at a low pH by

allowing a stream of $CO_2$ to bubble through it. Under these conditions the dye was concentrated in the chloroform layer and passed into the inner fluid.

How relatively efficient such physical models are as compared to the living cells is difficult to decide. It must be remembered that the indirect secretion of neutral red by living cells is at best a feeble process, and the great part that may be played by purely mechanical relations of the dye-containing fluid to the concentrating medium, chloroform, is shown by a simple experiment. If chloroform is shaken even gently with the alkaline dye-containing Locke's, so that it forms




TEXT-FIG. 3

isolated droplets (vacuoles) it concentrates within itself almost immediately all the dye of the fluid. If this dye-saturated chloroform is now gently shaken with acid dye-free Locke's solution the dye is almost instantly transferred to the aqueous medium. Such alterations in the physical relationships of the two phases make the model an infinitely more efficient mechanism for the transference of the neutral red from one medium to the other than are the living undamaged renal cells.

## DISCUSSION

The results of the experiments described in this and the preceding studies may be considered in their general and specific aspects. The

former has to do with their application to problems of cytological method, while the latter is concerned with their significance in an understanding of the secretory activity of the renal cells.

In their general aspect the experiments with neutral red would seem to confirm the validity of the extravital method as a useful adjunct to cytological investigation. Previous investigators (8) have examined the appearance of perfused organs, but never has the full means of the morphologist been applied with the directing idea that the results of such visual examination might be equally as valuable a part of the experiment as the findings of the physiologist. The essential matter is that neither aspect, functional or structural, shall be subordinated to the other in a correlation of simultaneous phenomena that is planned to give a complete view of the organ's total activity.

Our comparison of the manner of indirect secretion of neutral red in the extravital experiments with that observed in the living intact animal has shown a complete identity of processes. Not only do the conditions of the extravital experiment produce no artifacts in the cells, tissues or organs under examination by it, but under these conditions vital processes may proceed in exactly the same manner and by the same mechanisms, both structural and functional, as are observed in normal life. And this conclusion, taken in conjunction with our previous demonstration that the reactions of the tissues to noxious insults under the conditions of the extravital experiment are identical, both in structural change and functional response, to those observed in living animals (5, 2), would seem to establish the value of the extravital procedure among the methods of the new cytology (9, 10).

In the specific problem of renal secretion the extravital method has made possible by means of its controlled conditions an analysis of the manner of elimination of neutral red by the renal tubule cells. Secretion, which has hitherto been regarded as a unified single process, was found to be composed of two separate methods of elimination, each with its own peculiar mechanism. That under most conditions the two processes run concomitantly explains the earlier failures to recognize by the usual methods of examination the elimination as a composite process.

One of the methods of secretion is a prompt and efficient elimination

into the lumen of the tubule of the dye that has entered the cell from the blood vessel. This has been termed "direct secretion." The mechanism which controls it is the variation that occurs in the permeability of the two cell membranes, the one lying adjacent to the blood vessel, the other contiguous to the lumen of the tubule. Each of these membranes may be affected independently of the other by different factors. The permeability of the outer, or vascular, membrane depends on the balance between K and Ca ions of the fluid bathing it. The permeability of the inner, or lumen, membrane may be decreased by toxic substances such as potassium bichromate, without any significant change in that of the outer membrane. Depending as it does, therefore, on the reciprocal action of the two membranes that may function in the same or in an inverse sense, direct secretion is an easily disturbed process. Such disturbances account for the remarkably complex variations in the abnormal kidney's activity that are observed with the damaged organ in the secretion of neutral red and the absorption of substances from the urine that we have described in a previous study (2).

The other method of secretion has been called the indirect. It is characterized by the concentration of the dye within the cell to as much as 3000 times that of the perfusion fluid in the vessels. The elimination is a slow long continued process that in the living animal may be observed at least 20 hours after the original entrance of the dye into the cells. The distinctive mechanism concerned is an alteration in the mitochondrial apparatus of the renal tubule cells. These changes can be observed microscopically and consist of a disappearance of the filamentous mitochondria and the development from mitochondrial substance of large granulovacuolar structures which differ in their fixative and staining reactions from the original mitochondrial material although they still retain the characteristic reaction of the latter to Janus green. One of the most striking of these differences is the Gram-retaining power of the granulovacuoles as compared to the Gram-negative quality of the filamentous mitochondria. That these changes are not simply concomitant with the concentration of the dye, or the result of it, but that they are processes which determine in part secretion is evident from the fact that one of the changes is the acquirement by the granulovacuoles of the property of staining

with neutral red. This property is not possessed by the original mitochondria, and by it the dye is concentrated within the cell body during indirect secretion. In this sense the vacuoles function as Gurwitz' *condensoren* (11). If a replacement of filaments by vacuoles has already occurred in the renal cells as a previous response to some other stimulus, then these preexisting vacuoles function as condensors of the dye.

The indirect secretory process is not easily affected in its slow and constant elimination. No factors were found that definitely increased it in our experiments, and on the other hand it was observed to continue undisturbed when direct secretion had been almost completely eliminated by the action of toxic substances. It was found indeed that dead cells, killed by corrosive sublimate, still were able to concentrate within themselves a certain amount of the dye above that in the perfusion fluid, and the urine from such dead tubules, though identical to the perfusion fluid in other regards, contained some dye, in spite of the fact that little or no dye could have been present in the glomerular filtrate under the conditions of the experiment. The essential processes involved in indirect secretion are therefore not vital in any sense of the word, but depend on the chemical or physical constitution of the tubule wall that remains essentially unaltered even after its cells have been killed by the mercuric salt. That the dead cells are less efficient than the living may well be due to the fact that they are less permeable to the dye after coagulation of their protoplasm, as well as to the fact that after this severe damage the condensing substances are no longer arranged in the previously efficient emulsion-like pattern of the granulovacuolar droplets which present a very large effective surface. Further evidence that indirect secretion depends on simple physical or chemical processes is suggested by the fact that models which duplicate its action may be easily constructed.

Another illustration of the relation between the instability of direct and stability of indirect secretion is particularly well shown by the results of extravital staining with Janus green. It will be remembered that this dye enters the cell and is stored in the granulovacuolar apparatus but that it does not appear in the urine. Apparently its toxicity is sufficient to prevent direct secretion but inadequate to stop the concentration processes of the indirect secretion. That this inter-

pretation is correct is supported by the fact that in the combined extravital staining experiments after the perfusion of the kidneys with Janus green the direct secretion of neutral red was prevented, yet the indirect secretory process, both in its concentrating and eliminating phases, remained intact.

As has been stated previously, under the usual conditions that follow the administration of neutral red to an animal, the two methods, direct and indirect, are concomitant and so blended in their results as to give the appearance of a single process of elimination. There are, however, variations in the relative part played by either process, and one factor which determines this is the degree of concentration of the dye in the blood vessels. With low concentrations in the blood stream the indirect method predominates; with high concentrations the indirect, though present, is less marked while direct secretion is correspondingly active.

These facts concerning the secretory activity of the renal cells have been obtained by the study of the elimination of a substance foreign to the animal's economy. The question arises as to what physiological substance may be handled by the kidney in a similar manner. It is certain that some must be so handled, for the morphological evidence of the occurrence of the indirect secretory process can be seen in the tubule cells of animals living under native conditions, and the existence of the elaborate mechanisms of direct secretion can hardly be reconciled with the assumption that, existing, they are not used. It has been suggested that certain substances, such as phosphates and uric acid, may be eliminated in part at least by the tubules rather than by glomerular filtration and an examination of this possibility by the extravital method might seem a promising procedure. But the method is not at present applicable to the problem, for as we have used it the exact conditions that obtain in the animals' blood stream are not reproduced. Such conditions are necessary to determine if a substance, bound perhaps in the blood of the living animal, may or may not filter through the glomerulus. We have called attention to this point in a previous publication (12).

Although the evidence here presented need not be discussed in relation to the direct question as to what substances are eliminated in a similar manner by the living animal, certain indirect conclusions

may be helpful for further work. For example, recognition must now be taken of the fact that secretion by the renal cell is a composite as well as complex problem. As we have shown, one method of secretion may exist without the other and the complications that might arise in the interpretation of results unless this fact is recognized are evident.

It is the indirect method of secretion that interests us at present. It is a mechanism ideally fitted to effectively remove from the blood stream a non-filtrable substance which is present intermittently and in relatively low concentration. This is done by a process of storage within the kidney cells followed by a slow but ultimate elimination. Some of the normal deleterious end-products of the animal's metabolism may be included in such a category, but evidence seems to accumulate that they are eliminated more directly by glomerular filtration.

There is another category of deleterious substances, however, which must be eliminated and this includes both the end-products of disturbances in the organism's metabolism, that is of disease processes, and those toxic substances which fortuitously enter as casual contaminants of the food supply. We know that the kidney concentrates within itself and eliminates into the urine such grossly toxic exogenous substances as the heavy metals. The analogy with the indirect secretion of the relatively non-toxic neutral red is striking.

By such a concept indirect secretion as we have described it would become a protective mechanism in a stricter sense than can be applied to elimination of toxic metabolites. It might indeed be considered a pathological process, though the frequency with which all living organisms must call upon some method to free themselves from the results of such every day disturbances as we have mentioned, would make its occurrence almost a normal phenomenon. And it is of particular interest in this regard that pathologists have long sought to connect a certain frequently occurring pathological process, cloudy swelling, with the hyperactivity of cells, and that in the kidney the hyperactivity has been assumed to be a secretory one. Furthermore, the morphological evidence of cloudy swelling is a disturbance in the mitochondrial and granular apparatus of the cell and a typical part of this disturbance is the appearance of Gram-positive granules (13, 14). Such granules we found to be a constant evidence of a secretory activity in the renal cells unassociated with any toxic phenomena. These rela-

tions between the normal secretion and pathological changes in the cells are at present being examined by the extravital method.

## CONCLUSIONS

1. The elimination of neutral red by the renal epithelium is a composite process, consisting of a direct and an indirect secretion.

2. The mechanism controlling direct secretion is concerned with the permeability of the two cell membranes. These two membranes may be affected independently in the direction of either an increased or decreased permeability, with a corresponding increase or decrease in the elimination of the dye.

3. The mechanism controlling indirect secretion is concerned with the mitochondrial apparatus of the cell. By means of change in the form and constituent substance of its structures, the dye is concentrated within the cell and slowly eliminated.

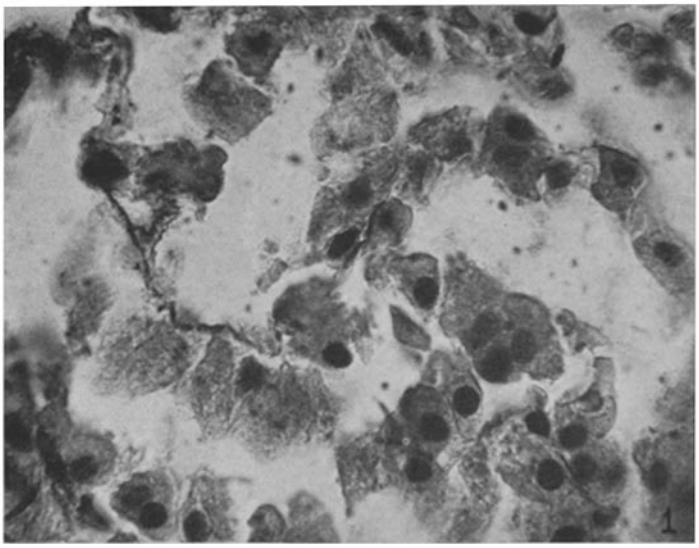
4. Direct secretion, depending on the condition of sensitive membranes, is easily disturbed. Such disturbances account for the wide variations in the elimination of dye observed in the functioning of abnormal kidneys. Indirect secretion, depending on the simpler factor of the solubility of the dye in the protoplasmic constituents, continues even when the cells are severely damaged.

## BIBLIOGRAPHY

1. Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A., *Tr. Assn. Am. Physn.*, 1931, **46**, 301.
2. Oliver, J., and Shevky, E., *J. Exp. Med.*, 1931, **53**, 763.
3. Sato, K., *Folia Anat. Japon.*, 1930, **8**, 391.
4. Gellhorn, E., *Protoplasma*, 1931, **14**, 28.
5. Oliver, J., and Smith, P., *J. Exp. Med.*, 1931, **53**, 785.
6. Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 995.
7. Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1932, **15**, 667.
8. Ssyssojew, Th., *Virchows Arch. path. Anat.*, 1924, **250**, 163.
9. Carrel, A., *Science*, 1931, **73**, 297.
10. Oliver, J., *Science.*, 1931, **74**, 174.
11. Gurwitz, A., *Arch. ges. Physiol.*, 1902, **91**, 71.
12. Oliver, J., and Shevky, E., *Am. J. Physiol.*, 1930, **93**, 363.
13. Pfister, M., *Beitr. path. Anat. u. allg. Path.*, 1905, suppl. 7, 525.
14. Fahr, Th., *Verhandl. deutsch. path. Ges.*, 1914, **17**, 119.

EXPLANATION OF PLATE 32

FIG. 1. Segment II of the kidney from the experiment of page 474 whose renal epithelium was killed extravitally by corrosive sublimate. Necrosis and desquamation are seen with pycnosis and absence of nuclear staining in the dead cells. Neutral red was nevertheless concentrated in these dead tissues. Magnification × 525.



(Oliver and Lund: Cellular mechanisms of renal secretion. I)