A MECHANISM OF CONSERVATION IN THE KIDNEYS OF THE WINTER FROG*

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A well known peculiarity of the kidneys of "winter frogs" has been recently emphasized by Höber (1) in his studies of renal function by the method of perfusion. During the months of October, November, December, and January, he finds it often difficult to obtain a proper response by the kidney. This is most noticeable in the excretion of water, some kidneys forming no urine at all in spite of copious and long continued perfusion of both the glomerular and tubular circulations. The only explanation of this phenomonon we have been able to find that is based on experimental data, is that offered by De Haan and Bakker (2). They have observed that winter frogs show a definite decrease in their ability to excrete dyes. Fluorescene, a readily diffusible dye which is ordinarily excreted completely in one day, is not eliminated by the winter frog in several days. In the summer frog this substance is found in the urine in a concentration two or three times that of the blood, while in the winter animal the concentration in the urine is often less than that of the blood. Trypan blue, a less diffusible dye, is found in the urine only in a very dilute state. These observations are interpreted as being due to an increased density of the glomerular membrane which prevents their filtration and consequent appearance in the urine.

In the course of a study on experimental nephritis in frogs made by the method of perfusion, we have come in contact with this problem and as certain of our experiments would seem to offer another explanation of these peculiarities in the behavior of the winter kidney, they are here reported.

Methods

All of the experiments described were done during the months of December, January, and February. The frogs, R. catesbiana, averaging 850 grams in weight, were obtained from Louisiana. Here the animals hibernate intermittantly from about December 1st to April 1st, as during this period two or three light freezes and several frosts may occur. These cold spells were particularly severe during this last winter, (1928–1929) and it was on frogs collected in this period that our experiments were done. On arrival at the laboratory, the frogs were kept in large tanks of running water at a temperature of about 15°C. and were used within a week or ten days. Under these conditions they were quite active, and had all the appearance of summer frogs.

The method of perfusion which we have used is similar to that of Höber with the important modification of the perfusion pressures, our arterial pressure equaling 45 centimeters of water, while the venous pressure was 20 centimeters. The importance of this modification we have discussed in a previous paper, and will mention again in conclusion.

The perfusion fluid was the modified Locke's solution of Barkan, Broemser and Hahn (3) buffered to a pH of 7.4 and properly oxygenated. Glycocol to a concentration of .50 percent, and glucose to .05 percent were also added to the solution.

The details of the operative procedure have been previously described and will therefore not be repeated. Canulae from the perfusion flasks were placed in the anterior abdominal vein, thus supplying the renal-portal system, and into the aorta just below the junction of the two thoracic branches. Uretral cannulae were placed in the ureters, and the urine collected in measured periods of time. The samples of urine obtained were expressed as to volume in cubic centimeters per hour, the presence or absence of sugar tested with Benedict's Solution, the electrolyte content determined with a Christiansen ionometer, the reading being expressed as a percent concentration of NaCl, and any dye present determined quantitatively with a Dubosq colometer. All these determinations may be made with considerable accuracy, as the samples obtained are large, the normal volume of urine being from 8 to 10 centimeters per hour.

EXPERIMENTAL

Before detailing the results of perfusion of the kidneys of winter frogs, it is necessary to describe those obtained by similar procedures with the frog in summer when the organs of the animal are in a state of maximum activity.

Under these conditions, with the method described above the volume of urine from the two kidneys varies, as stated above, from 4 to 10 cubic centimeters per hour.

Sugar is normally absent from the urine, though it may be present in the first fifteen minute sample collected. Any persistent appearance of it indicates, as Höber has shown, a damaged kidney, and is usually accompanied by an abnormal increase in the amount of urine.

The electrolyte content of the urine under normal conditions is considerably lower than that of the perfusion fluid. The lowest figures which we have obtained are 20 percent of that of the Locke's solution, while as an upper limit, we have taken 50 percent. If it approaches nearer than this figure to the salt content of the perfusion fluid, sugar is usually present and the volume is large; in other words, there is evidence of what Höber has shown must be interpreted as tubular damage.

In a previous article (4) it was shown that the two dyes, neutral red and phenol red, are excreted in different manners. The former is eliminated almost entirely through the tubules, while the phenol red is excreted in by far the greater part through the glomeruli.

These two dyes were used in the perfusion fluid at a concentration of 12.5 mg. per 1000 cubic centimeters for neutral red, and 20 mg. per 1000 cubic centimeters for phenol red. The rate of excretion of phenol red by the normal kidney under these conditions varies from .5 to 1.2 mg. per hour. In spite of this variation in rate between the kidneys of different animals, abnormalities can be readily recognized as a rule by the inconstancy of the findings. An abnormal low excretion decreases progressively as the perfusion proceeds to a very low figure and is associated with other evidences of kidney damage, such as a high salt content, or the presence of sugar.

The concentration of the dye in the urine as compared to that of the perfusion fluid (Höber's "concentration factor") varies greatly, affected as it is by the volume of water excreted. A concentration factor as low as 200 percent may be found in urine which is otherwise normal, the rate of excretion of the dye remaining within normal limits due to a large volume of urine. With small volumes of urine as high a factor as 3000 percent may be obtained. On the other hand, when this figure falls as low as 100 percent, the urine contains sugar, a high salt content, or some other evidences of kidney damage are present.

The rate of excretion of neutral red also varies considerably with different kidneys. From .45 mg. to 1.5 mg. per hour have been found under normal conditions, but here again the constance of a normal kidney is easily distinguished from the progressive fall observed with abnormal organs. The concentration factor, as in the case of phenol red, is dependent largely on the rate of water excretion. A factor as high as 7500 percent has been observed.

The Perfusion of Normal "Winter Frog" Kidneys

In the first five periods of Figure 1 is shown a typical experiment illustrating the effect of perfusion on the kidney of the winter frog.

The dye in the Locke's fluid was phenol red. It will be noticed that although in the first period the volume of water was within normal limits for summer conditions, 9 cubic centimeters per hour, during the first five periods there is a gradual and progressive decrease to 2.4 cubic centimeters per hour. The arterial perfusion flow was practically constant during this period, so that the decrease in volume can not be explained by any failure of the perfusion method. A faint trace of sugar was present in the first period, but disappeared in the next sample and remained absent during the remainder of the experiment. The salt content in the first period was 50 percent, decreasing to 45, and finally to 40 percent of the concentration in the perfusion fluid in period 5.

The phenol red, as in most of the experiments to follow, was introduced first into the tubular circulation, and then into the glomerular system. This was to test our previous findings, that the excretion of this dye is chiefly through the glomerulus. As is seen in periods 1 and 2, only a very low rate of excretion resulted, .02 and .04 mg. per hour. The rate rose however at once to .16 mg. per hour when it was led to the glomeruli, an eight fold increase. This rate is, however, still far below the usual figure for a summer frog, and in the following periods 4 and 5 it decreased progressively to a final figure of .07 mg. per hour. The highest concentration factor attained was only 115 percent.

The experiment described thus far, differs strikingly from the results of perfusion of a summer frog in that there was a gradual and progressive decrease in the excretion of all the elements of the urine. The water decreased, sugar disappeared, and salt became less, as did also the rate of excretion of phenol red. The concentration factor of the latter was, however, greater than 100 percent. In other experiments similar results were obtained, in some the volume of urine decreasing to less than .1 cubic centimeter per hour, and in other cases the volume from the beginning of the perfusion was so low that the perfusion might be said to be ineffective.

Viewing these findings in the light of De Haan and Bakker's theory that the winter frog's kidney is characterized by a decreased filtration of the urinary constituents through the relatively impermeable glomerular membrane, it is difficult to explain by such a mechanism

the concentration of the dye in the urine above that of the perfusion fluid. In other experiments, this concentration factor of phenol red rose to such figures as 410, 520, 540, 840, and even 1000 percent as the volume of water decreased. Obviously the concentration mechanism is still active in the winter frog's kidney and this fact led to the idea that perhaps all of its peculiarities might be explained by the assumption of an increased absorptive activity on part of its tubules.

The hypothesis is easily tested. The absorptive function of the tubules may be depressed, as Höber has shown by anesthesia with
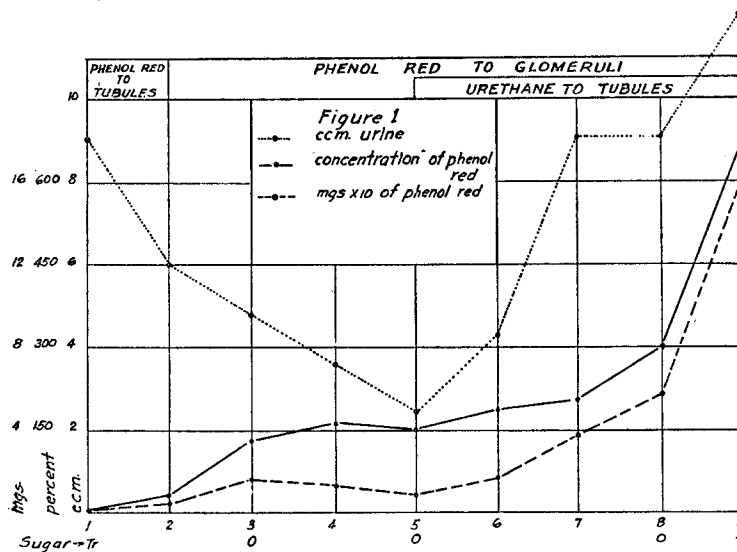

FIG. 1

urethane, and if our assumption is true, such a procedure should cause the kidney of the winter frog to form a urine which is similar in amount and constituents to that of the summer frog. The second half of figure 1 (periods 6, 7, 8, and 9) shows such an experiment.

Beginning at the removal of sample 5, urethane in a concentration of 2 percent was added to the tubular perfusion fluid, and in periods 6 to 9 all the evidences of a depression of tubular function as described by Höber and observed by us in our previous article developed.

The rate of water excretion increased steadily until it reached 12 centimeters per hour, a figure which equals the output of the summer frog. An equally striking increase is noted in the output of phenol red. The rate of excretion was

doubled in the first period following the anesthesia and progressively increased until in the ninth period it reached a figure which equals even the maximum figure attained by summer frogs, that is, 1.6 mg. per hour.

That these increases in water and dye output are associated with a failure of tubular absorption, is seen by the changes in salt and sugar excretion. Absorption of salts remained active until the eighth period, when the concentration rose to 50 percent of that of the perfusion fluid concentration and finally to 60 percent in the last period, at which time a trace of sugar also appeared in the urine.
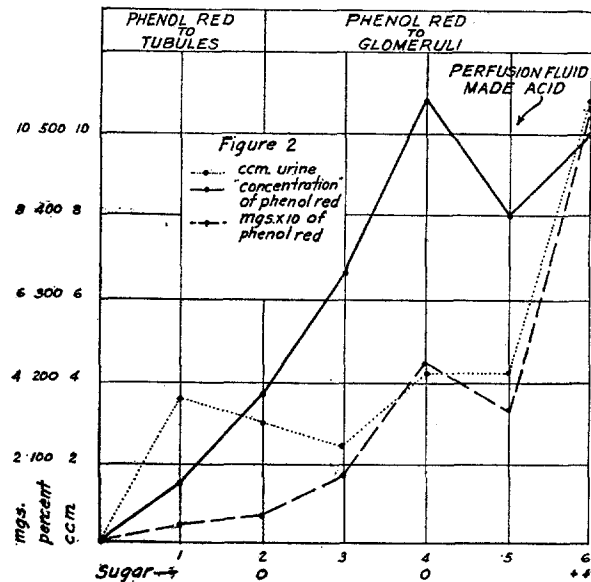

FIG. 2

These results are not the peculiar effect of the anesthetic, for any damage to the tubular epithelium which produces a depression of its function results in similar effects. In figure 2 the depression of tubular absorption was produced by frank damage to the epithelium with an unsuitable acid perfusion fluid. Under these conditions the scanty winter urine, poor in dye content, not only increased to values in water and dye which might be considered normal for a summer frog, but continued to show a decrease in tubular absorption until a point of frank abnormality was reached.

The perfusion was started with Locke's solution at the normal pH of 7.4 and the usual amount of phenol red. The volumes of urine in the first 5 periods were

low or moderate in amount, and contained no sugar. While the phenol red was administered to the tubules, (periods 1 and 2) the rate of excretion was very low, .08 mg. per hour, and increased over five fold to .44 mg. per hour, when it passed to the glomeruli (period 4). This figure is however lower than that of the average summer frog and begins to fall in the 5th period. At this time the perfusion fluid to the tubules was made acid to a pH of 5.9. The epithelial cells were immediately damaged, and their absorptive activity destroyed as evidenced by the appearance of sugar in the urine. As a result, the rate of water output rose to what might be considered a normal figure for the summer frog, 10.8 cubic centimeters per hour, and the rate of excretion of phenol red to the high rate of 1.6 mg. per hour.

These experiments support strongly the idea that the apparent "inactivity" of the winter frog's kidney is the result of an increased activity on the part of its absorbing tubules rather than that there is any decrease in the process of glomerular filtration. Both water and dye are present in sufficient amount in the tubular lumen and are excreted readily when the tubule cells are prevented from absorbing them.

In considering the latter part of the two experiments just described, in which the excessive absorbing activity of the tubules has been depressed either by anesthesia or by frank damage to the tubule cells, one is struck by the fact that not only is a large amount of dye excreted, but also that although the normal concentrating mechanism, absorption of water, has been depressed, never the less the dye is still found in considerably higher concentration in the urine than in the perfusion fluid. Evidently then, some water is being absorbed from the glomerular filtrate or there is an added source of dye output in these periods, or both of these possibilities exist.

Although it is impossible to answer such an involved question in any completely satisfactory quantitative manner, it can at least be shown that the second named factor may well play an important part in the concentrating process. The following experiment (Fig. 3) shows that under certain conditions an added source and mechanism exists for the excretion of phenol red which may not function to any significant degree under normal conditions of renal activity.

Clear Locke's solution was supplied to the tubules while phenol red in the usual concentration was introduced into the glomerular circulation. The dye was excreted in high concentration, (780 percent) and at the moderately high rate of

56 mg. per hour. As is typical of the winter frog, the next period showed a drop to about one half this figure, .25 mg. per hour, the concentration factor was lower and the volume of urine decreased. At the end of this period the dye containing fluid of the glomerular circulation was replaced with clear Locke's solution, so that no dye was being supplied to the kidney. Nevertheless, the rate of dye excretion remained practically constant, .22 mg. per hour and since the volume of water had continued to decrease, the concentration factor was somewhat higher, 620 percent. The following period also showed a high rate of excretion, but in the 5th and 6th periods the lack of dye supply began to make itself apparent, and the rate of excretion and the concentration factor of the dye fell to a low figure.
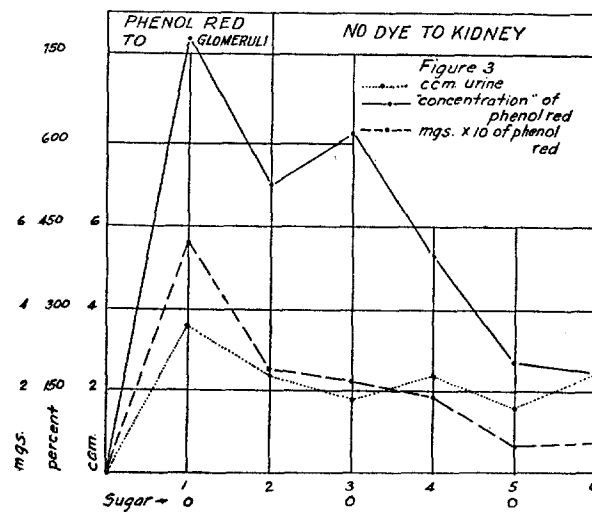

FIG. 3

For one half hour, therefore, the kidneys had maintained the rate of excretion and concentration of the dye without the usual source of glomerular supply. Since no other tissues were included in the perfusion system, it is obvious that the source of this excreted dye must have been the kidney itself.

It has been shown by many observers that a storage and concentration of phenol red occurs in the cells of the renal tubule (Marshall and Crane (5)) and Hayman and Richards (6) have proved by direct observation that when introduced into the capsular space of the glomerulus the dye appears within the tubule cells. Richards and Barnwell have furthermore shown (7) that although normal tubule cells are able to maintain a higher concentration of phenol red than

exists in the fluid in which they are immersed, when damaged this is no longer the case. With these facts in mind, it seems clear that the dye which had been stored during the first periods of the experiments described was subsequently liberated from the tubule cells and excreted during the period of dye free perfusion. Such a source and method of dye excretion is particularly important in the winter frog, for the kidneys of such animals whose absorbing mechanisms are extraordinarily active, are especially rich in stored dye, so that when damaged by the urethane as in experiment 1, a considerable amount may be added by liberation from the tubule cells to that amount which is being eliminated by glomerular filtration.

Evidence has been presented which has been interpreted as indicating that the kidneys of winter frogs conserve water, salt, sugar, and, when present in the urine, dye, by a process of excessive absorption from the urine within the renal tubule. It has been shown by Scheminzky (8) and by the writers, that neutral red is excreted almost entirely through the tubule epithelium. Its manner of excretion is therefore different from that which we have found for the excretion of phenol red. In the kidneys of the winter frogs which are so active in conservation by their mechanism of absorption, one m'ght expect a similarly conservative action on the part of the excretory function of the tubules. The following experiment shows this to be the case (Fig. 4).

Neutral red was introduced into the tubular circulation. In the first period it was excreted at a moderate rate (.45 mg. per hour) and in high concentration (735 percent), but in the next two periods (2 and 3) there was a marked fall in both rate of excretion and concentration. The final figure of .03 mg. per hour and a concentration factoj of 100 percent are far below any figures obtained with normal summer frogs. The volume of urine decreased and the absorption of salt was active as its concentration in the urine was only 40 percent of that of the perfusion fluid in the 3rd period.

The kidneys of the winter frog therefore excrete neutral red to a lesser degree than those of the summer frog and the conservative mechanism of excessive absorption is complemented by a decrease in the excretory function of the tubular epithelium. The experiment was now continued for the examination of another point of interest.

It has been shown that phenol red which has been absorbed by the tubule cells from the lumen of the tubule may be subsequently excreted back into the lumen when the concentration of dye in the urine is low or when the tubule cells have lost their absorptive power as a result of damage.

The findings in the fourth and fifth periods show that there may be an analogous excretion of stored neutral red from the tubule cells. At the end of period 3 the perfusion fluid containing neutral red was replaced by dye free Locke's solution. The kidney was now receiving no dye, yet during the next period the rate of elimination of neutral red remained nearly as high as the preceding period and the urine was more concentrated in dye than the perfusion fluid. By the sixth period both rate of excretion and concentration factor had fallen, evidently due to the exhaustion of the available neutral red which had been stored in tubule cells.[1]

It will be noticed in the sixth period that the volume of urine was small, 1.2 centimeters per hour, and the salt concentration was especially low, only 30 percent of the concentration of salt in the perfusion fluid. This is strong presumptive evidence that we are dealing with typical winter kidneys, that is, that their absorptive mechanisms are very active. Nevertheless, this point was tested with phenol red as in our previous experiments. This dye was supplied to the glomeruli during the 7th period and in the 8th period was excreted at a moderate rate, .24 mg. per hour and in moderately high concentration, 495 percent. At the beginning of the 9th period tubular absorption was depressed by administration of urethane in a concentration of 2 percent to the tubules. The rate of water elimination immediately rose to 10.6 centimeters per hour and there was an increase in the rate of phenol red excretion to .66 mg. per hour. The absorptive mechanism of the tubules had indeed been so depressed by the anesthetic that the concentration of salt rose to 60 percent of that of the perfusion fluid and sugar appeared in the urine.

In the same winter kidneys, therefore, an excessive absorptive activity and a decreased excretory function was demonstrated in the tubular epithelium.

DISCUSSION

From the experiments described above, the conclusion has been drawn that in winter frogs there exists a profound alteration in the function of the tubular apparatus of the kidneys. This alteration is manifested by an increase in the activity of their resorptive processes and a decrease in their excretory functions. The method of demon-

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[1] Scheminzky has described the storage of neutral red in the tubular epithelium and we shall later discuss this point more fully in a study of experimental nephritis by means of vital staining.

stration of these facts included the study of substances such as water, salts and sugar, which are normally present in the blood and urine, but the most striking demonstration was made with the dye, phenol red.

In a previous article (4) we have shown why we believe that the principal method of phenol red excretion is through the glomerulus.

While this article was in press, there appeared a study by Scheminzky, working in Höber's laboratory, who from somewhat similar experiments came to a different conclusion. He found, as did we, that neutral red was excreted by the tubules and only slightly by the glomerulus, but contrary to our conclusions, he believes that phenol red is also excreted principally by the tubules.

It is impossible to adequately discuss this important and lengthy paper, and we shall only attempt to call the reader's attention to certain points which we feel should be especially noted in comparing the results of the two investigations.

In the first place the type of experimental animal is different. Scheminzky used winter animals, and such material judged in the light of De Haan and our present paper is obviously not the most satisfactory for the study of the method of normal dye excretion. As a result, the volumes of urine, and amounts of dye excreted are very small. In one emphasized experiment .19 cubic centimeters per kidney per hour is given as a normal volume and in another .86 cubic centimeters is cited as an example of diuresis due to tubular narcosis. The technical difficulties of quantitative NaCl and phenol red and qualitative sugar determinations all on a thirty minute sample under these conditions are manifest, especially when dye output must be expressed in millionths of grams.

Of even greater importance, however, is the difference in perfusion technique in Scheminzky's and our study. In most of his experiments he used a pressure of 18 and 9 centimeters of water for the arterial and venous systems and under these conditions the venous flow was greater than the arterial.[2] In our previous article

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[2] In discussing the improbability that fluid from the venous circulation has reached the glomeruli Scheminzky on page 667 gives a theoretical calculation as evidence of the impossibility of such an occurrence. Without going into the details which he presents we wish to point out that although it is correct that at least 9.1 centimeters of dye containing perfusion fluid must be assumed to have reached the glomeruli from the venous circulation in his hypothetical case, it is not at all necessary to assume that this was mixed in the glomeruli with the 40 cubic centimeters of dye free fluid which flowed through the aortic circulation. The flow through the glomerular capillaries may in fact have been purely venous and

we have pointed out that in order to cause the kidney to function in a normal manner pressures of 45 and 20 centimeters are required. Detailed experiments have shown that such pressures produce no abnormalities in the formation of the urine by either the glomeruli or tubules and that only with them will perfused kidneys secrete a volume of urine which is comparable with what Adolph (9) has shown to be the normal rate of water excretion.[3]

As a result of these seasonal and technical differences, and also because our frogs were perhaps much larger, our volumes of urine are larger and may be considered as normal in regard to water excretion. The determinations of its constituents are made with ease and the results of dye excretion are expressed as milligrams.

So much for general differences in method. In the specific experiments certain differences should also be noted. In contrasting the excretion of phenol red by the tubules and by the glomeruli Scheminzky used different animals to test each part of the renal unit. The dye was found in the urine in concentrations higher than in the perfusion fluid after both tubular and glomerular administration and these results on different animals were then compared by averages and by "specific concentration" and "Leistungs" factors rather than by rates of excretion, for these rates varied in different animals. In our experiments the comparison of the excretion of either dye by the two parts of the kidney, glomerulus and tubule, was done in consecutive periods of a single experiment in the same animal and the contrasting results expressed as simple rates, i.e., mgs. per hour.

In regard to the effect of narcosis of the tubules on the excretion of phenol red, Scheminzky states that in five of twelve experiments although the anesthetic produced its typical effect on all the other urinary constituents, it did not depress the rate of excretion of the dye, as is required to support his hypothesis of tubular secretion. Doses of from two to five times the size which produced these results were required to regularly produce a depression of dye elimination. This is ex-

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in amount equal to any fraction of the total venous flow, the arterial fluid being shunted around the glomerular tuft by way of the arteriae rectae to join the intertubular capillaries. Such anastomoses have been shown by Nussbaum and Hayman to mention only the first and last of a long series of investigators. Under these conditions, which are as proper an assumption as that of Scheminzky, it would be a simple matter to account for the total amount of dye found in the urine as a result of venous rather than arterial perfusion of the glomerular tuft.

[3] As a matter of fact the pressures used by us are even less than the maxima that have been observed existing in the living frog's circulation by many other observers. Hayman gives a figure of 60 centimeters of H_2O as a possible normal aortic pressure, the average being 37 centimeters. Our venous pressure could doubtless be reduced, and this would lessen the danger of a contaminating venous perfusion of the glomerulus, but we have selected the pressure given above as proper to insure a complete circulation around the tubules of the entire kidney.

plained by the assumption that the secretory function of the tubule is more resistant to narcosis than the absorptive process.

Another explanation is, however, possible and that is that the larger doses of anesthetic affect more of the renal apparatus than the tubule. In a series of experiments on experimental nephritis in the frog to be published later, in which attempts were made to cause selective damage to various parts of the kidney, great difficulty was encountered in damaging any one part of the renal unit without affecting to some degree the other elements. The glomeruli, the tubules, and especially the vascular system are all susceptible to damage when strong toxic agents are administered even with great care to any one portion of the kidney, and under these conditions of wide spread damage its function is altered in such a complex way that it is impossible to judge as to the part played by any single structure in the production of the total effect.

Our experiments on the winter frogs have also shown an accessory mechanism for the elimination of phenol red by the kidney. As the following table shows, although the increases after tubular damage in rates of water and dye excretion are in most cases of similar magnitude, occasionally the dye elimination is increased to a far greater degree.

Increase in water elimination	Increase in dye excretion
2.5	2.7
8.2	7.3
2.3	2.2
2.5	3.2
5.	22.0

The last figure of this group, which is from an experiment in which there was a long period of dye absorption before the administration of the anesthetic to the tubules, it would appear that there is some other source of dye than that responsible for water output. As our experiment 3 shows, this added source is doubtless the dye which has been absorbed from the lumen and stored in the tubule cells. An "anomalous secretion" of such stored dye is apparently possible under unusual conditions, such as, for example, when the concentration of dye in the lumen of the tubule falls far below that in the tubule cells, or when these cells are damaged and no longer able to hold it in concentrated form. Here again the phenomenon is best observed in winter frogs for their tubule cells are more highly charged with dye as a result of their excessive absorptive activity.

Our experiments on the activity of the winter frog's kidney also add a further demonstration of the difference in the manner in which the kidney handles neutral red and phenol red.   In such animals with neutral red the concentration factor is usually low when the rate of excretion has fallen to a low figure.   With phenol red, the concentration factor is always greater than 100 percent, and often considerably larger, even though the rate of excretion is small.   This difference also may be explained by our previous theory of tubular and glomerular excretion of the two dyes.   The method of concentration of neutral red is a single mechanism in that it is a function of the tubule alone,
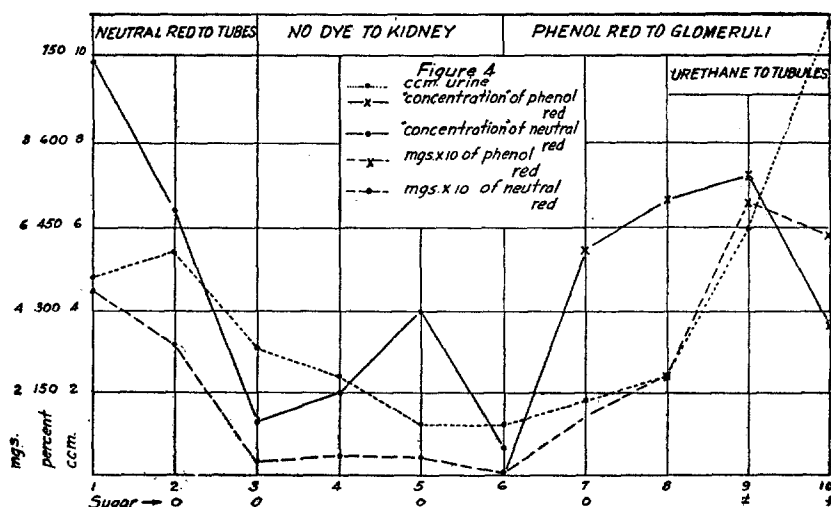

FIG. 4

namely tubular excretion of dye and tubular absorption of water. In the case of phenol red the concentration of the dye is the result of the activity of at least two mechanisms, glomerular excretion and tubular absorption of water and to a lesser degree of dye.   An increased activity of the latter may therefore reduce the amount of dye excreted into the final urine without decreasing its concentration, since it is reasonable to suppose that water is absorbed more readily than the dye.   The method of "anomalous secretion" of phenol red which we have just described may also aid in producing the result of a high concentration but under these conditions the rate of excretion is apt also to be high.

CONCLUSIONS

1. A method of conservation has been demonstrated in the kidneys of winter frogs.

2. The mechanism of this conservation is an increase in the absorptive function and a decrease in the excretory activity of the tubular epithelium.

3. The excessive absorptive process may be depressed by various means.   Such a depression in the tubular activity is followed by large increases in the rate of excretion of water, salts and, if present in the urine, phenol red.

4. Further evidence is thus obtained which supports the theory that phenol red is excreted chiefly through the glomeruli.

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