A COMPARISON OF THE METHOD OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE MAMMALIAN KIDNEY*

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During the last decade a considerable addition to our knowledge of kidney function has been made by the study of renal activity in the comparatively simple mesonephroi of the amphibia. The kidney of the frog in particular has been subjected to investigation by a long series of observers who have used methods, such as direct observation (Richards (1)) and perfusion (Höber (2)), to which the mammalian kidney does not readily lend itself. As a result facts have been ascertained directly concerning processes which up to the present time have been investigated in mammals only by indirect methods. These latter methods have of necessity been based largely on hypothesis and have produced chiefly theory, so that if the phenomena observed directly in the frog's kidney could be demonstrated in the mammalian kidney a considerable advance would be made. It has been tacitly assumed by many that an analogy between the two types of animals is a proper one, but since little if any direct evidence has been produced to support such a contention the present study is offered as an attempt at such experimental confirmation.

The possibility of the investigation arose in the following way. In a recent study of the manner of excretion of phenol red and neutral red by the perfused frog's kidney an interesting contrast was found in the mechanism of elimination of the two dyes (Oliver and Shevky (3)). Phenol red was found to be excreted chiefly through the glomeruli

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while the more colloidal neutral red was eliminated almost entirely through the tubules. It would seem, therefore, that with such a striking dissimilarity in the manner of excretion of these two dyes by the frog's mesonephroi that experiments on the excretion of the two dyes by mammals, under varying conditions, might cast some light on the question as to the possibility of analogous contrasting mechanisms of elimination in mammalian kidneys.

Another point of interest also presents itself in the study of the excretion of the two dyes by mammals. Phenol red has long been used as a clinical test of kidney function. If it should prove to be the case that neutral red is excreted in a different manner and by a different mechanism by mammals it is possible that it might serve as an adjunct to the phenol red test of the kidney.

With these points in mind the excretion of the two dyes has been studied under various conditions in frogs and rabbits.

Frog Experiments

Methods.—In the frog experiments the following methods were used. The kidneys of large Rana catesbiana of from 850 to 1000 gms., weight were perfused by the method which we have previously described in detail (3). Very briefly, the perfusion fluid is a modified Locke's solution containing sugar which is led to the kidneys by the renal arteries, thus supplying the glomeruli and at the same time by the renal portal venous system which perfuses the tubular circulation. Pressures of 40 and 20 cm. of water are used on the artery and vein respectively.

Under these conditions the kidney produces a urine which is normal in amount and in its constituents. The volume varies from 7 to 10 ccm. per hour, urea is concentrated, salts are diluted, sugar is retained and colloidal substances such as proteins and gum arabic do not pass into the urine. The two dyes phenol red and neutral red, if added to the perfusion fluid in concentrations of around 15 mgs. per 1000 ccm. of fluid, are concentrated many times and as we have stated are excreted by the glomeruli and tubules respectively.

The analytical methods used in studying the urine were as follows: For sugar, Benedict's qualitative solution, for the dyes a Duboscq colorimeter and for the salts a Christiansen ionometer, the results of this latter determination being expressed as per cents of NaCl.

Experimental.—In figure 1 is shown the experimental results of perfusing the frog's kidney with a Locke's solution containing urea and phenol red in concentrations of 20 mg. and 500 mg. per liter re-

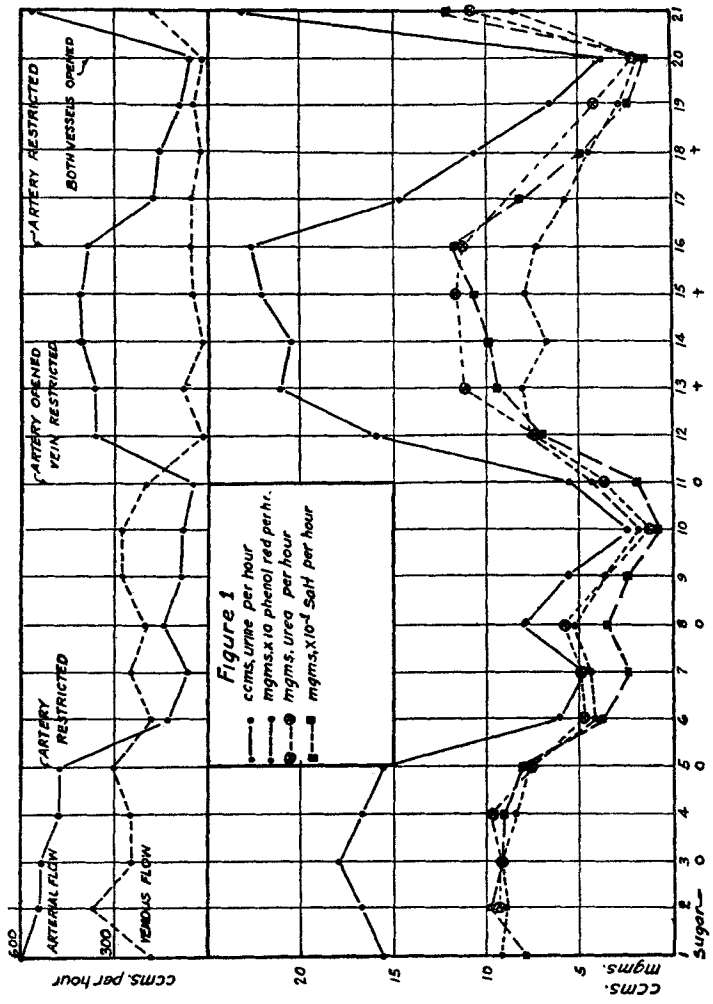
ARTERY RESTRICTED
BOTH VESSELS OPENED
ARTERY OPENED
VEIN RESTRICTED
ARTERY RESTRICTED
ARTERIAL FLOW
VENOUS FLOW
600
300
ccms. per hour
Figure 1
ccms. urine per hour
mgms.x10 phenol red per hr.
mgms. urea per hour
mgms.x10⁻¹ salt per hour
20
15
10
5
ccms.
mgms.
Sugar

FIG. 1

spectively. These substances were present in the fluid which supplied both the glomeruli and the tubules, so that they were available for excretion by both of these elements of the kidney. In the upper portion of the chart is seen the outflow for the two circulatory systems after the fluid had passed through the kidney.

For the first five periods the perfusion was allowed to proceed in a normal manner. The volume of urine obtained was somewhat higher than that usually obtained, averaging about 16 ccm. per hour but the amount was fairly constant during the entire five periods. No sugar was present in the urine. The concentration ratio, i.e., the concentration of the phenol red in the urine as compared to the concentration in the perfusion fluid was also fairly constant, averaging from a 250 to a 300 per cent increase. The rates of excretion, expressed as mgs. per hour, of urea phenol red and salt were also constant. They averaged 10 mgs. per hour for urea, 1 mg. per hour for phenol red and 100 mgs. per hour for salts.

At the end of the fifth period the flow through the glomeruli was restricted by clamping the rubber tube which leads to the arterial cannula. This resulted in a marked decrease in the arterial outflow, while the circulation through the venous tubular system remained unaffected. Very marked changes were noted at once in the urine. The volume decreased to 6 ccm. per hour, a fall of 62.5 per cent. The rates of excretion of urea, phenol red and salt also fell to approximately one-half their former value.

These conditions continued through the next five periods, during which time the glomerular circulation remained low. The venous circulation remained adequate, however, for at no time was there sugar in the urine.

The interpretation of these findings in the light of our previous findings (3) is plain. A lessened supply of urea, salt, phenol red and water to the glomeruli produced a corresponding decrease in the rate of excretion of these substances and this in spite of the fact that these substances were being administered in excess to tubules which, judged by their ability to absorb sugar, were entirely normal. The major source of excretion of all these substances must have been the same therefore, and this source the glomeruli.

The converse experiment was now performed. At period 11 the

glomerular supply was increased by removing the constriction on the artery and the venous supply to the tubules decreased by restricting the flow to the vein. There was an immediate reestablishment of the former levels of excretion of water, salt, phenol red and urea. In the 13th period the effect of the tubular insufficiency became evident for sugar appeared in the urine, a sign of lack of tubular absorption. For this same reason a moderate "tubular diuresis" resulted, for it will be seen that in periods 13 to 17 although the arterial flow through the glomeruli is less than during the periods of normal perfusion (1–5), nevertheless the rate of water excretion is considerably higher. The same is true of the rates of excretion of urea and salts. In the case of phenol red the previous normal level was not exceeded in this particular experiment.

Under the conditions of this phase of the experiment, therefore, that is with adequate glomeruli supply and with frankly damaged tubules, a result of "anemia" and lack of oxygen, the kidney excreted phenol red, urea and salts at a rate equal to that of the normal kidney. The latter two substances were even excreted at an increased rate, since these substances as has been shown by other methods, are absorbed from the lumen of the tubule when the tubular epithelium is functioning normally.

The remainder of the experiment repeats the previous demonstration of the effect of glomerular "anemia." It will be seen that the rates of excretion of urea, salt and phenol red vary together and follow the rate of excretion of water, and that all these rates depend on an adequate glomerular supply. In contrast to the predominantly glomerular excretion of phenol red figure 2 shows a similar experiment in which the kidneys were perfused with Locke's solution containing urea and neutral red in concentrations of 12.5 and 500 mg. per liter. As in the first experiment both urea and dye were administered to tubules and glomeruli simultaneously by artery and vein and were therefore available for excretion by both of these parts of the renal unit.

In the first period 15 ccm. per hour of urine was excreted. The rate of urea excretion was 15.9 mg. per hour and the rate of neutral red excretion 4.3 mg. per hour. Salts were eliminated at the rate of 48 mgs. per hour. The concentration ratios of the various substances

have not been charted as their variations throughout the experiment are of much less significance than the rates of excretion. During the first period the dye was 23 times as concentrated as the perfusion fluid and the urea three times as concentrated.

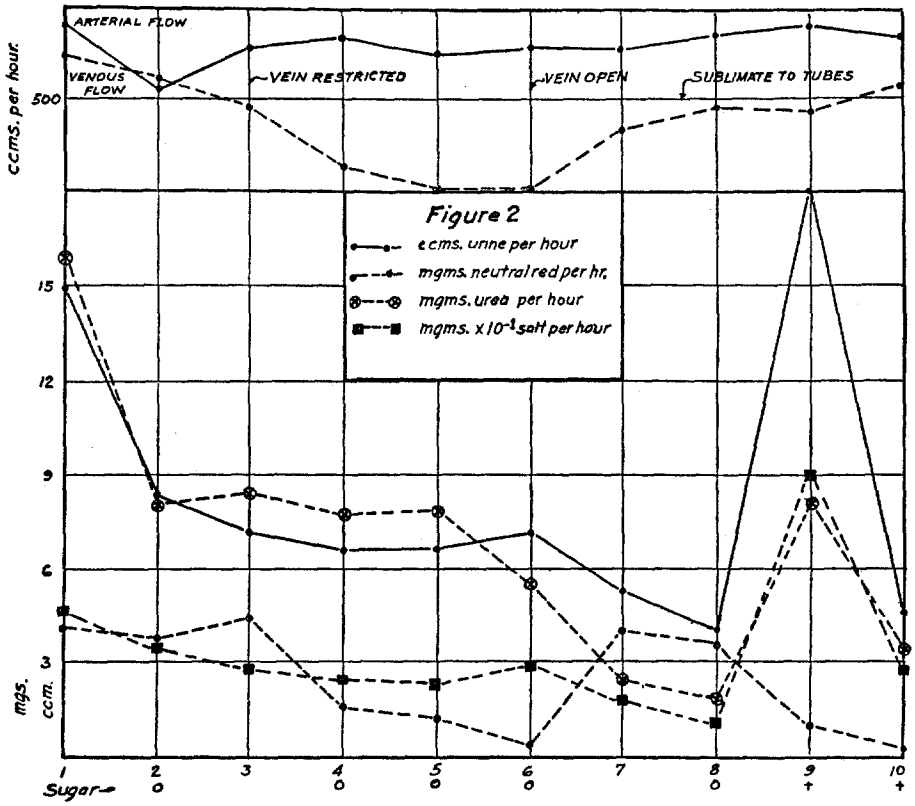

FIG. 2

In period 2 the arterial flow through the glomeruli was lessened and, as a result, the volume of urine excreted fell to 8.6 ccm. per hour. The rate of urea excretion followed this drop to 8.4 mg. per hour, as did the rate of salt excretion (34 mg. per hour), these results being similar in nature to those of the previous experiment. A striking difference is noted in the rate of excretion of the dye. Instead of falling, as did the rate of phenol red excretion in the first experiment,

its rate of excretion was unaffected by the change in glomerular elimination as it remained approximately equal to the previous period. Period 3, under the same conditions, was essentially the same as period 2.

At this point the supply of dye to the tubules was decreased by restricting the flow of perfusion through the vein. In this experiment, however, no actual damage to the tubule cells was produced. No diuresis developed nor did sugar appear in the urine as a result of lack of tubular absorption. The glomerular circulation remained as in the previous periods and the rate of water, urea and salt excretion continued through the next three periods (4, 5 and 6) essentially unchanged. But again the striking contrast in the excretion of the two dyes is seen, as the rate of neutral red elimination instead of following that of urea as did phenol red in the previous experiment, fell progressively to a final figure of .56 mg. per hour.

At the end of period 6 the vein was reopened and the tubules supplied with the former amount of neutral red. Since the tubules, as just noted, had been undamaged in this experiment the rate of neutral red excretion immediately rose to its former figure, 4.1 mg. per hour.

At the end of period 8 a new procedure was introduced into the experiment. 10 ccm. of $\frac{1}{4000}$ corrosive sublimate was administrated to the tubules during a 10 minute interval at low pressure. We have described elsewhere the result of such a procedure (3). It is followed by all the results of tubule damage, i.e., diuresis and escape of sugar into the urine and, as we will show in a later study, is frequently followed by a repression of urine, probably the result of vascular damage. Our interest here is only in how the dye excretion varies under these unusual conditions.

As will be seen in period 9 a diuresis developed, 18 ccm. per hour, and large amounts of sugar appeared in the urine. As in the previous experiment when the tubules were damaged by "anemia," the rate of urea excretion increased, as did that of salt elimination. But the rate of neutral red excretion instead of accompanying this rise as did phenol red in the first experiment, fell at once and finally reached the extremely low figure of .07 mg. per hour.

In periods 10 and 11 the typical sublimate repression of urine developed and with the fall in water excretion there went the usual fall in rates of urea and salt output.

These two experiments illustrate again the antithesis in the excretion of the two dyes. One, phenol red, behaves as if it were excreted principally by the glomeruli; the other, neutral red, as if the tubules were its chief source of elimination. But for the experiments with mammals which are to follow, the important contribution of these experiments is that the rate of urea excretion may be used as a standard to which the dye excretions may be compared.* It is by taking advantage of this fact that the following study of the methods of excretion of the two dyes by the mammalian kidney is made possible.

Mammalian Experiments

In following the elimination of dyes and urea by the perfused frog's kidney only the actual rate of excretion of these substances need be followed, for one of the most important factors in determining this rate, namely the concentration in the perfusion fluid, is constant. In the experiments on living rabbits designed to determine whether or not the methods of excretion shown to exist in the amphibian kidney can be transferred to the mammalian kidney, the maintenance of such a state is impossible, since the perfusion fluid here is the plasma of the circulating blood. Experience with the excretion of other substances, such as chloride (4), phosphate (5, 6), creatinine (7, 8) and particularly urea (9), has shown that in the absence of definite knowledge of the behavior of a substance that not the rate of excretion alone but this rate in relation to the plasma concentration or the excretory ratio: $\frac{\text{Urine rate}}{\text{Plasma concentration}}$ should form the basis of study and comparison. In the choice of conditions under which to observe this ratio experience gained in the study of urea excretion was again called upon. The rate of urea excretion by the kidney is influenced by a variety of factors other than the blood urea concentration. Constancy of these factors is best obtained (10) when there is a marked stimulation of renal tissue or when as Addis has pointed out (11) the

* We do not wish to discuss at this time the mechanism of urea excretion, reserving this for a later communication. In these experiments its method of excretion resembles that of phenol red and its chief source of elimination is the glomerulus.

conditions are such that it seems reasonable to assume that all of the renal elements have been wakened to activity. It seemed highly probable that other factors than the dye concentration of the plasma may accelerate or inhibit phenol red and neutral red excretion and, in that case, it is to be expected that a balance of these factors would also best be obtained during a heightened renal activity. This was attained by the administration of urea and large quantities of water. During the ensuing diuresis simultaneous observations of the serum dye concentration and the rate of dye excretion in the urine were made.

Methods.—Healthy male rabbits were chosen for these experiments. When observations were terminated the animals were killed with ether and whenever the kidneys were not entirely normal the experiment was discarded. Except for the low urine volume experiments the general procedure was identical for every animal. No food was given for fifteen hours before the experiment commenced. Three hours before the first catheterization, when observations were begun, 40 ccm. per kilo body weight of a 5 per cent solution of urea was given by stomach tube and every hour thereafter until the experiment was ended 40 ccm. of tap water per kilo was administered in the same manner. The urine was obtained by catheter at approximate intervals as recorded in each experiment. After each catheterization the bladder was thoroughly washed with a known volume of distilled water. Arterial blood was obtained from the heart without an anticoagulant at the middle of each period of urine collection. Analyses were performed on the serum. There is scarcely a demonstrable difference in the urea or dye concentration of serum and paraffine plasma from the same sample of blood. Urea in both the serum and urine was determined by a urease and aeration method. Phenol red and neutral red in the serum and plasma were determined by the addition of NaOH in one case and HCL in the other and comparison after the necessary dilution with known standards in a Dubosq colorimeter. The results are all expressed as milligrams excreted per hour and milligrams per 100 ccm. of serum.

Comparison of the Excretion of Phenol Red and Neutral Red

Numerous experiments were performed with varying degrees of success in comparing the excretion of phenol red with neutral red. The phenol red experiments were usually successful while with the more colloidal neutral red difficulties were encountered. At the normal pH of rabbit plasma this dye in comparison with phenol red is relatively insoluble and in attempting to obtain higher and higher plasma concentrations some animals were killed in so-called "ana-

phylactoid" shock. Whatever the quantity of dye injected by far the greater part rapidly leaves the blood stream and as compared with phenol red only relatively low plasma concentrations can be

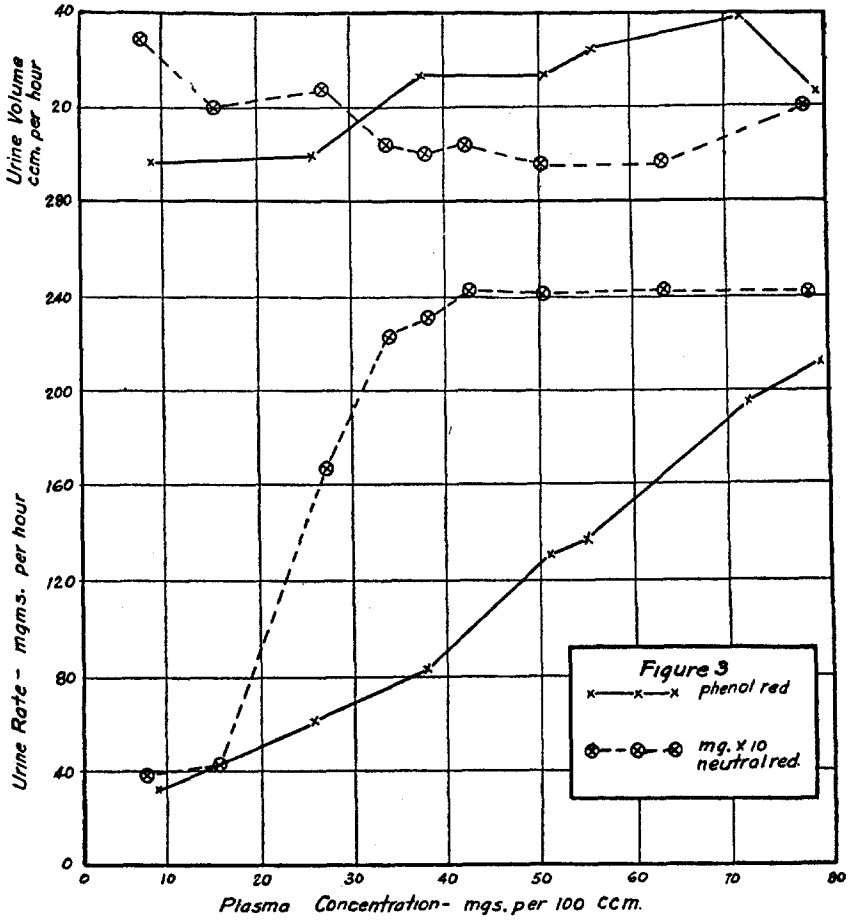

FIG. 3

obtained. Three or more observations were obtained for both dyes in 14 rabbits. In every case the main results were the same. A typical experiment follows:

A male rabbit weighing 2500 grams was given water and urea as described and 3 hours later 500 mgms. of phenol red were given intravenously in 2 per cent

solution. Ten minutes after this the bladder was washed out and the first urine collection commenced. Urine was collected at half hour intervals and serum specimens obtained at the middle of these periods. The observations are plotted in figure 3. Six days later this rabbit was again given urea and water. Thirty minutes before the first urine collection was commenced 100 mgms. of neutral red in 1 per cent solution were injected intravenously. Fifty mgms. of the dye were given every 30 minutes thereafter until the end of the experiment. Urine collections were made at half hour intervals and serum specimens obtained as before.

In figure 3 the neutral red observations have been compared with the phenol red excretion figures. There can be little question that neutral red is excreted in a manner very different from that of phenol red by the mammalian as well as the amphibian kidney. The rate of phenol red excretion in the urine is directly proportional to the plasma dye concentration while the rate of neutral red excretion bears no constant relation to the concentration of neutral red in the plasma except that a maximum rate of excretion independent of the plasma dye concentration is reached while the latter is still at a low figure.

An experiment reported by Marshall and Crane (12) which with other data led these authors to conclude that phenol red was secreted by the tubules of both the amphibian and mammalian kidneys requires some comment at this point. In comparing the rate of phenol red excretion to the plasma phenol red concentration in a dog these investigators failed to find the direct relationship which we have described but instead obtained a curve resembling our own neutral red observations. An examination of the protocol of their experiment reveals possible reasons for the discrepancy between their results and ours. They collected urine specimens as small as 1.0 cc. and even with the ureters cannulated these volumes are too small to give any degree of accuracy to urine collections from a dog's kidney when the tubule and pelvis dead space is taken into consideration. Another factor which would nullify their unsupported observations is the time interval used for urine collections. In no instance did this exceed the very short period of 3 minutes. Another and perhaps the most important factor contributing to the nature of their results is the fact that blood samples were taken not at the middle but at the beginning of the 3 minute periods of urine collection. Presumably this was done to correct for the urine volume of the kidney dead space and would conceivably have done this had the urine volume remained the same throughout the observations. This however was not the case and the deviations from a direct plasma concentration—urine rate relationships are in a general way those which might be expected from the variation in the urine volume.

Comparison of the Appearance Time of Phenol Red and Neutral Red

Another marked difference in the excretion of phenol red and neutral red which is not shown in the type of experiment illustrated in figure 3 is the interval between the commencement of the intravenous injection of the dye solution and the time of its appearance in the bladder urine. In comparison with phenol red neutral red was always very slow in making its appearance in the urine. By continuous washing of the bladder during a marked diuresis the dye appearance time was determined with a fair degree of accuracy in a number of experiments. In the experiments where it was recorded neutral red appeared in the bladder urine 3, 5, 3, 9, 12, 15, 16, 16, 17 and 22 minutes after the intravenous injection was begun. The appearance time apparently bore no relation to the dose. In one case phenol red required over 4 minutes to reach the urine but in more than a dozen other experiments the appearance time was uniformly less than 1 minute. If we assume an analogy between the excretory mechanisms of the frog and rabbit kidneys in so far as these dyes are concerned it is easy to visualize the reason for the marked difference in the appearance time of these two dyes. Phenol red would pass through in the glomerular filtrate almost immediately on reaching a *fully active* kidney and would soon be seen in the urine. On the other hand it would seem reasonable that neutral red, if secreted by the tubules, requires a measurable length of time to be removed from the plasma into the cells and passed into the urine in any amount.

Comparison of Phenol Red and Urea Excretion

Phenol red and urea are excreted in a similar manner by the perfused frog's kidney. Proof that this was also true in the rabbit would contribute additional evidence indicating an analogy in the mechanisms of renal function of these two types of animals. The following experiment is typical of a group of five in which excretion of phenol red and urea were simultaneously compared.

A male rabbit weighing 2.5 kilos was treated as previously described and 15 minutes before the collection of urine was begun the animal received 15 cc. of 5 per cent phenol red solution intravenously. Urine was collected over eight

30-minute periods and blood samples taken at the mid-point of each of these periods. The results form figure 4.

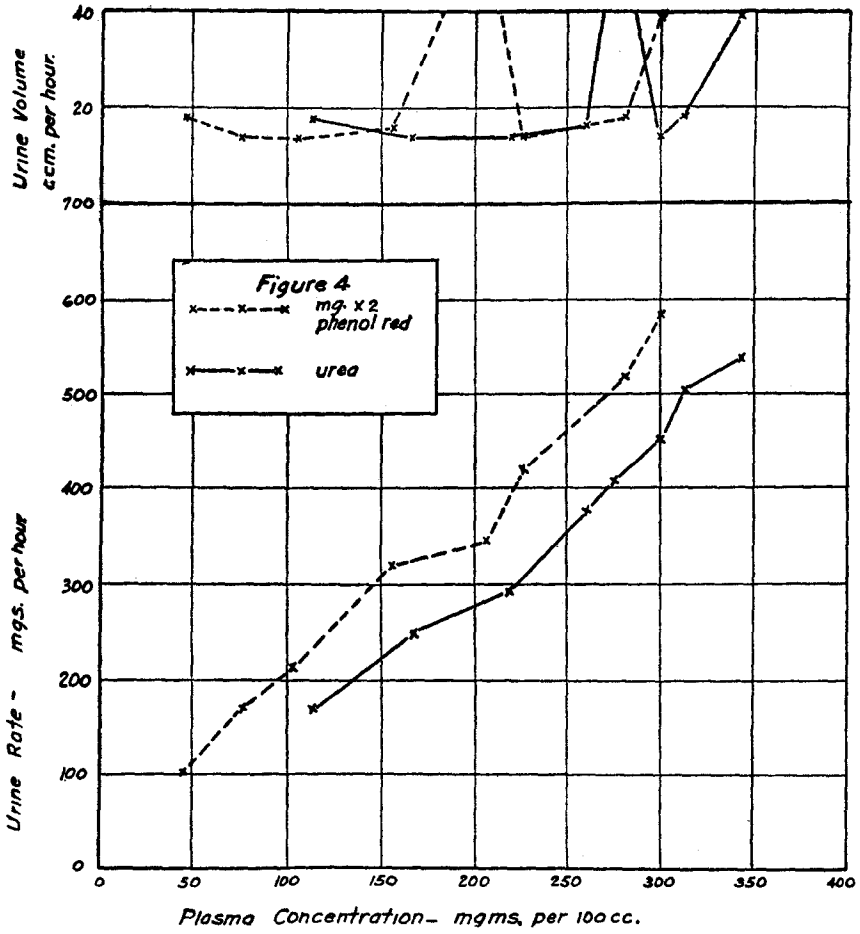

FIG. 4

Phenol red then is excreted by the mammalian kidney in the same way as urea, that is, in both cases the rate of excretion in the urine is directly proportional to the plasma concentration. In the particular experiment cited here the ratios: $\frac{\text{Urine Rate}}{\text{Plasma Concentration}}$ are rather small for both urea and phenol red in relation to the size of the rabbit

and at postmortem the kidneys were found to be normal but considerably smaller than usual. The difference between the ratios, that of phenol red being higher than that of urea, is a constant finding which will be discussed in another communication.

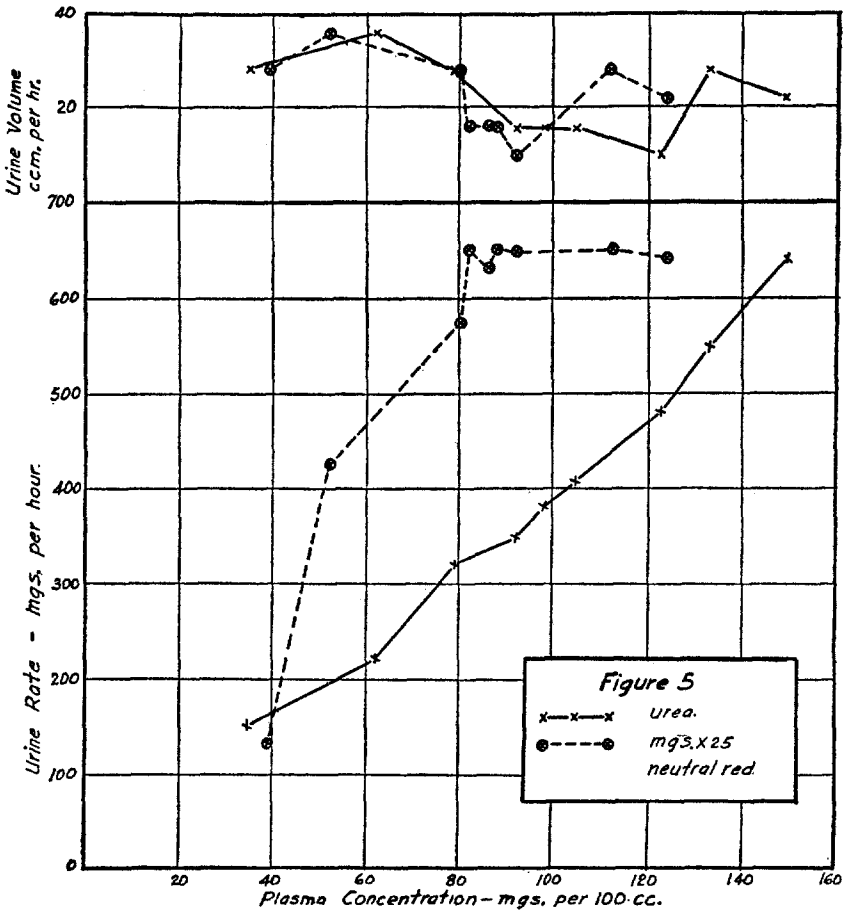

FIG. 5

Comparison of Neutral Red and Urea Excretion

In contradistinction to phenol red, neutral red is excreted by the amphibian kidney in a manner unlike urea. Likewise neutral red

and urea are excreted differently by the mammalian kidney. A typical experiment, one of three in which this point is demonstrated, follows:

A 3.5 kilo male rabbit received water and urea as described previously. Neutral red was given intravenously as in experiment 3, 175 mgms. being injected in 1 per cent solution each half hour. Nine 30-minute urine collections were made, a sample of serum being obtained at the middle of each one.

The results in figure 5 show the usual relationship under these conditions for urea, the urine rate being directly proportional to the plasma concentration. The excretion of neutral red on the other hand bears no consistent relation to the plasma concentration at low levels of the latter and at higher levels the rate remains constant despite further increases in the concentration of dye in the plasma.

Relation of Phenol Red Excretion to Urine Volume

Rowntree and Geraghty (13) and other observers (Marshall and Kolls (14)) have held that the rate of phenol red elimination by the kidneys is independent of the fluid output. If this were so the excretion of this dye would be different in this respect from urea. Although no direct relationship between the rate of urea excretion and the urine volume has been demonstrated it has been shown (15, 16) that both the rate of urea excretion as measured by the ratio: $\frac{\text{Urine Urea Rate}}{\text{Blood Urea Concentration}}$ and the urine volume tend to vary with the degree of renal activity. Low urine volumes and low urea ratios are generally found at low degrees of renal activity and increase with the degree of renal activity until the urea ratio is at a maximum when the urine volume alone may continue to increase. If phenol red and urea are excreted by the same mechanism the relationship of the excretory ratios to the urine volume should be similar. That this is so in the frog's kidney has been shown in the experiments already described and the experiment which forms figure 6 demonstrates that this is also the case in the mammalian kidney. These observations were obtained as follows:

A 3.5 kilo male rabbit was kept without food or water for 15 hours. At the end of this period 3 ccm. of a 5 per cent phenol red solution were injected intra-

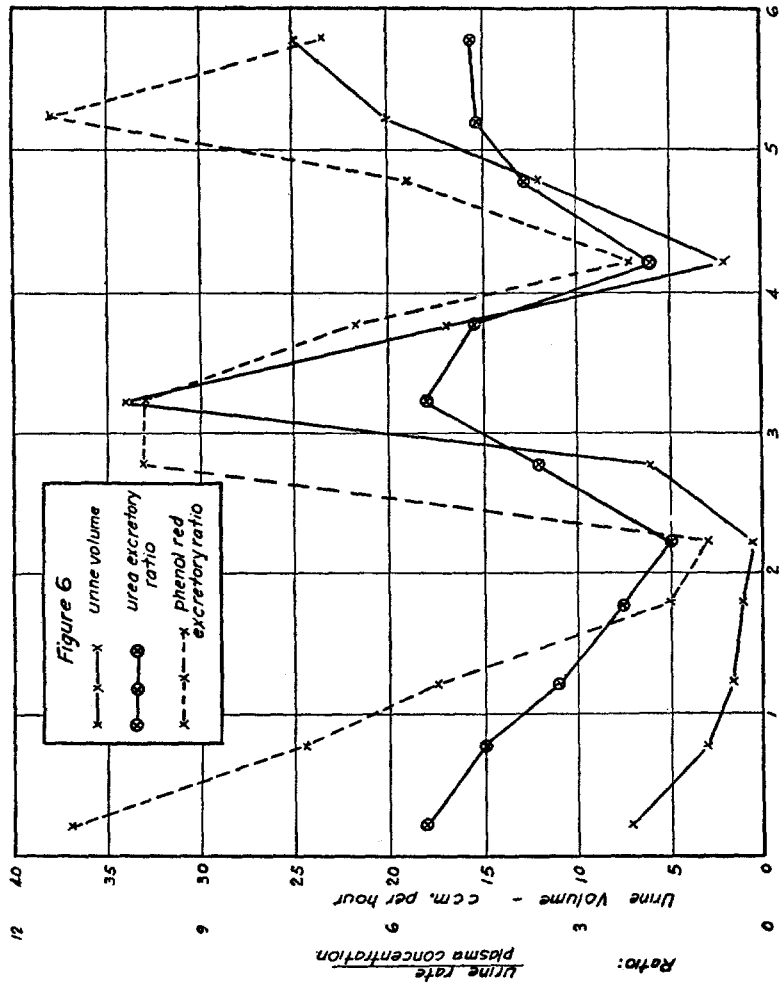
Figure 6
urine volume
urea excretory ratio
phenol red excretory ratio
Time in hours
Urine Volume - c.c.m. per hour
Ratio: urine rate / plasma concentration

FIG. 6

venously and ten minutes later the bladder was drained and washed with several small known volumes of distilled water all of which was returned. During the ensuing 6 hours 12 half-hour urine collections were made, the bladder being washed out at the end of each one with four 10 cc. portions of water. A sample of blood was obtained at the middle of each urine period. One and a half hours after the first urine collection was commenced 3 cc. more of 5 per cent phenol red solution were given by ear vein. At 2.5 hours 100 cc. of distilled water were given by mouth. Five cc. of 2 per cent urea and 2 per cent phenol red were given intravenously at 3 hours and 10 cc. at 4.5 hours. At 5 hours the rabbit received 10 cc. of 20 per cent creatinine and at 5.5 hours 15 cc. of 20 per cent creatinine and 0.2 per cent phenol red by intravenous injection.

The observations in this experiment show very clearly that the rate of phenol red excretion, just as does the rate of urea excretion, fluctuates with the urine volume under conditions of less than full renal activity. This result is at variance with the view which is generally held that no changes in urine volume are associated with changes in phenol red excretion. The reason for this is that previous observers have neglected to take into consideration the plasma dye concentration and have dealt not with the rate of excretion but with the percentage of an injected quantity which appeared in the urine in an arbitrary period.

SUMMARY

A direct examination with the method of perfusion of the excretion by the frog's kidney of phenol red and neutral red has shown that the dyes are eliminated in different manners as a result of different mechanisms. The former is excreted in much the greater part by the glomeruli; the latter by the tubules. Urea is excreted in a manner similar to phenol red.

The indirect examination of the function of the mammalian kidney by means of excretion ratios has shown a like contrast between the manner of elimination of the two dyes, and here again was found a similarity in the manner of excretion of phenol red and urea.

This would seem to be as close an examination as can be made with our present methods of experimentation of the question of the mechanism of the excretion of these substances by mammals. As the facts stand they constitute strong presumptive evidence that in mammals and amphibia the like results have arisen from like causes,

phenol red and urea being eliminated chiefly through the glomeruli in both instances while neutral red is excreted principally through the tubules.

CONCLUSIONS

1. There is a difference in the manner of excretion of phenol red and urea from that of neutral red by the frog's kidney.

2. This difference is due to differences in mechanism of excretion, the elimination of the former being by the glomeruli and the latter by the tubules.

3. There is a similar difference in the manner of excretion of these substances by the mammalian kidney.

4. It is inferred that this similar difference in manner of excretion is due to a similar difference in method of elimination, and that in mammals too, phenol red is eliminated chiefly through the glomeruli and neutral red through the tubules.

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