A COMPARISON OF THE MANNER OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE FROG'S KIDNEY*

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Since the beginning of modern physiological experimentation on the function of the kidney, the study of the manner of its excretion of dyes has proved a fruitful method of attack on the problem of the activity of its complex elements. The literature covering the subject matter of these studies has grown to vast proportions, but needs no review at this time as it has been frequently and thoroughly discussed.

Our interest in the manner of excretion of two dyes, phenol red and neutral red, arose in the course of an investigation of the problems of experimental nephritis which we had produced in frogs and which we studied by means of the newer methods devised by physiologists for the examination of their problems. Among these methods is that of perfusion of the kidney.

The perfusion of the amphibian kidney offers many advantages over similar procedure with mammals. Among the first to use the method extensively were the English observers, Bainbridge and his coworkers (1) and more recently Höber and his pupils (2) have published a long series of studies on the normal function of the frog's kidney. Among their many observations they found evidence indicating that the dye cyanol was not excreted by the tubule but by the glomerulus.

Our original problem was the testing of damaged kidneys, and among other methods that of perfusion was used. One of the reagents

15

employed was phenol red.   As another line of attack, vital staining with neutral red was employed.   It soon became evident that the kidney handled the two dyes in different ways and in view of the conflict in opinion of various workers on the subject it was thought worth while to extend our investigation to a more detailed study of the manner in which the two dyes are excreted by the normal kidney. Our findings seem to warrant consideration for themselves as distinct from their bearing on the more specific problem of the abnormal kidney.   They are, therefore, presented separately.

Methods

The method of perfusion which we have used is essentially that devised by Barkan, Broemser and Hahn (3) and elaborated by Höber.

Large bull frogs were used (*R. catesbiana*), averaging 800 gm. in weight, as this allows the collection of considerable amounts of urine, at times as high as 20 cc. or more per hour, and a correspondingly accurate determination of rates of excretion of various substances.   The perfusion fluid was that of Barkan, Broemser and Hahn, consisting of a modified Locke's solution buffered· to a pH of 7.4 and oxygenated with the proper mixture of CO_2 and O by means of their apparatus.   Glycocol in a concentration of .52 per cent was added according to the recommendation of Höber (4) and glucose in a concentration of .05 per cent. The frogs were pithed, the viscera with the exception of the liver and heart, lungs and kidneys removed, with ligation of all severed vessels, and canulae introduced into the anterior abdominal vein—thus supplying the renal portal venous system, —and into the aorta just below the union of the two thoracic branches.   In order to restrict the perfusion as much as possible to the kidneys the aorta was then tied below the renal arteries, the large veins to the legs ligated and the vena cava cut to allow the escape of the perfusion fluid which had passed through the kidney.   The perfusion pressure was always kept as low as compatible with a free flow of fluid through the vessels, as a rule the venous pressure being 20.0 cm. of water, the arterial 40.0 cm.   In both ureters were placed collecting canulae and the urine was removed in 15 minute periods.

Certain routine data were collected in all experiments.   The rate of water excretion in cubic centimeters per hour was recorded and the presence or absence of sugar was tested with Benedict's solution.   The total electrolyte content was followed by means of a Christiansen ionometer, giving the relative concentration of salts which was expressed as a percentage of NaCl.   The concentration of dye in the urine was determined with a Duboscq colorimeter and a known standard. In our findings only one typical example is given, but for each experiment sufficient confirmatory experiments were performed to leave no doubt of the result.

EXPERIMENTAL

The excretion of phenol red when supplied to the tubules alone was compared with its excretion when supplied only to the glomeruli.

In these experiments phenol red in a concentration of 20 mg. per 1000 cc. of perfusion fluid was supplied to the tubules by way of the vein, while Locke's
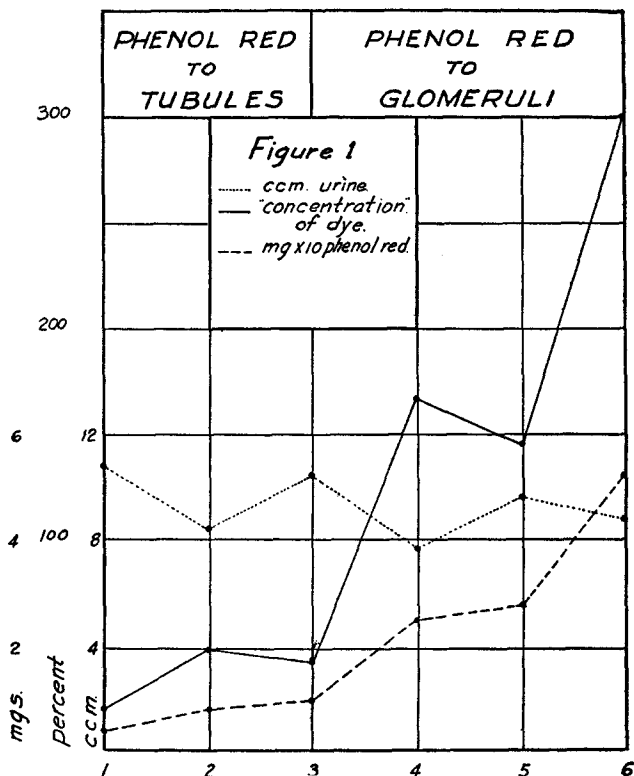

FIG. 1

solution free of dye was at the same time perfused through the glomerular tufts by way of the aorta and renal arteries. The pressure in the latter was about 45 cc. of water as compared to only 20 cc. in the venous tubular system. Before the addition of the dye the kidneys were perfused as routine with clear Locke's solution through both systems for two 15 minute periods at least to be sure that they were working in a normal manner. This was assumed to be the case, in accordance with Höber's findings (2), when the urine was free of sugar and its salt

content was not more than 50 per cent of that of the perfusion fluid. After the introduction of the dye, the 15 minute collections were tested in the routine way and the presence of dye noted and its concentration determined. The results are shown in Fig. 1.

During the preliminary periods the urine was sugar free and its salt content 40 per cent of the perfusion fluid. On the introduction of the phenol red into the fluid which went to the tubules no significant change was noted in any of its constituents. The presence of the dye was just perceptible to the eye and on determination was found to be about 50 per cent of that in the perfusion fluid the rate of excretion being .08 mg. per hour. After 3 periods the manner of dye administration was reversed, the concentration previously given to the tubule being led to the glomerular circulation by way of the aorta and renal arteries, while the tubular system of capillaries received clear Locke's solution by way of the renal portal vein. In other experiments dye was added to the glomerular fluid as described above, while the tubular system continued to receive the dye containing Locke's solution with no significant difference in result. The effect of this change in the method of administration of the dye is shown in the 4th period of Fig. 1. The excretion of water, sugar and salt remained practically unaltered but there was a sudden and immediate increase in the dye excretion from a previous level of 50 per cent of the perfusion fluid to 300 per cent. Expressed as rates of excretion, the rise was from .08 mg. per hour to .52 mg. per hour, or a six fold increase. In this particular experiment the increase in the amount of dye excreted by the glomeruli as contrasted to the amount put out by the tubules was not as large as that usually obtained. A summary of nine experiments gave an average increase of twenty-six times, the range of variation being from six to fifty fold.

Evidently phenol red when supplied to the glomeruli of the frog's kidney is excreted at a rate which may be as much as 50 times as great as when it is supplied to the tubules. Some dye, a small amount indeed, is excreted however when the supply is to the tubules alone, and this finding might be interpreted in one of two ways. Either this slight excretion is through that part of the kidney directly supplied by the renal portal system, that is through the tubules, or the fluid from the tubular venous capillaries passes over into the glomerular circulation in small amounts and the dye contained in it is excreted by the glomerulus.

We see no way to devise an experiment that will furnish an absolute control to the first possibility. It is a simple matter, however, to show that the second is not only possible but highly probable, for it has been demonstrated by others that fluid certainly does pass from the tubular to the glomerular circulation, especially if the proper rela-

tions between the pressures in the two systems are not maintained. The following experiment illustrates this point (Fig. 2).
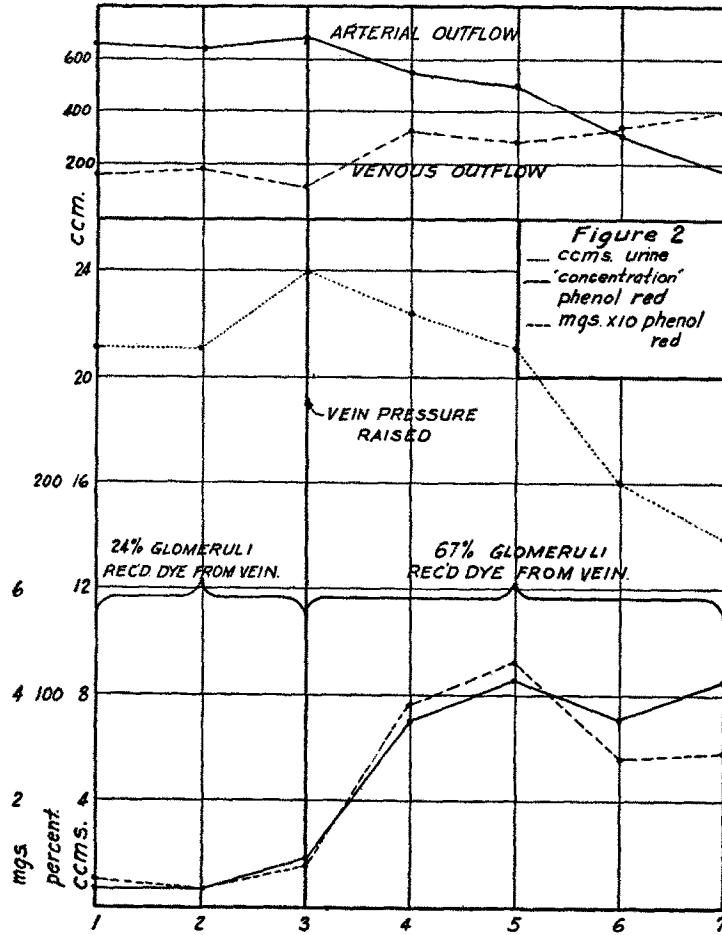

FIG. 2

After two preliminary periods in which the kidney functioned normally, 20 mg. of phenol red and 2 cc. of India ink suspension were added to 1000 cc. of Locke's solution in the bottle supplying the venous system, while the arterial bottle which supplied the glomeruli contained plain Locke's solution. The height of the two bottles was the usual one, 20 cm. for the venous tubular system and 45 cm. for the arterial glomerular bed. The out-flow from the two bottles through the two

systems is shown in the graph, the arterial flow being greater than the venous flow, which is the normal relation for perfusion by the method.

As will be seen from the graph, an average concentration of dye equal to only 10 per cent of that of the perfusion fluid was observed, while these conditions prevailed (Periods 1, 2 and 3). This equals a rate of excretion of .04 mg. per hour. The left kidney was then quickly removed with ligation of its vessels so that there would be no leakage during perfusion and fixed in 10 per cent formalin.

The pressure in the venous capillaries was now increased by 10 cm. Thereupon the flow through the venous capillaries increased considerably and though the rate of water excretion remained approximately the same, the concentration of dye rose to 90 and then 110 per cent of that of the perfusion fluid, or a rate of .45 mg. per hour (Periods 4 and 5). This figure is 10 times the rate of the preceding periods. The right kidney was then removed and fixed. Sections of the two kidneys were prepared and examined. The number of glomeruli which contained carbon particles, that is, which had received fluid and dye from the tubular circulation, in the case of the left kidney which had excreted .04 mg. per hour of the phenol red was 24 per cent of the 150 counted. In the right kidney where the rate of dye excretion had risen to .45 mg. per hour, 67 per cent of the glomeruli counted contained carbon as evidence of an increased flow from the tubular venous systems.

This experiment leaves no doubt that some dye reaches the glomeruli from the venous circulation, that the amount of such dye increases as the pressure on the fluid in the latter system is increased, and that concomitantly with this increased passage of dye to the glomeruli there is an increased rate of dye excretion by the kidney. We therefore believe it likely that the small amount of dye which was observed to be excreted in our first experiment during the period of tubular perfusion was evidence not of tubular excretion of the dye but of an unavoidable imperfection in the method of experiment. Some leakage from the tubular to the glomerular system cannot be avoided so that glomeruli receive the dye, and as the second part of the first experiment shows, it is readily excreted through them.

The excretion of neutral red when supplied to the glomeruli alone was compared with its excretion when supplied only to the tubules.

Before detailing the results of our experiments on the excretion of neutral red by perfused kidneys, it is necessary to test by experiment the statement of certain investigators who claim that neutral red is not excreted by the kidney of the living frog.

10 cc. of saline containing .5 mg. of neutral red was injected into the dorsal lymph sac of a frog whose bladder had been emptied by a catheter. The animal was kept in a glass jar where any urine passed might be recovered. After 60 minutes the bladder was again emptied. 2 cc. of urine was obtained containing .05 mg. of the dye. It follows that 10 per cent of the amount injected was recovered.

The same method of experiment was then applied to rabbits. Five mg. of neutral red was given intravenously in 20 cc. of 3 per cent NaCl solution. After
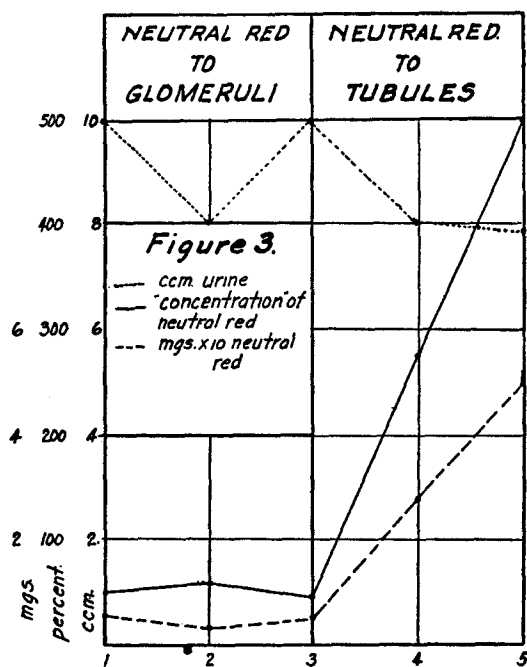

FIG. 3

1 hour the animal was catheterized again. One rabbit excreted 16 per cent, another 12 per cent of the dye. The average excretion of 8 more animals was 21 per cent with a range from 14 to 31 per cent. As there seems no doubt that neutral red is excreted by both the amphibian and mammalian kidney, the manner of its excretion was further examined by the method of perfusion.

The first perfusion experiment to be described is similar in its method to the first one described with phenol red. Other experiments relating to vital staining of the kidney with the neutral red had led us to suspect that it might be handled in a different manner than is phenol red. For this reason this dye was first introduced into the glomerular system by way of the artery and after a certain number of

periods was then supplied to the tubular apparatus by way of the renal portal vein.   Fig. 3 shows the result.   During Periods 1, 2 and 3 in which the dye reached the glomeruli in large amount, only small amounts appear in the urine, averaging about 50 per cent of the concentration in the perfusion fluid, an average rate of .06 mg. per hour.   The method of administration was then reversed, the neutral red now going direct to the tubules by the vein and the glomeruli receiving clear Locke's from the arterial bottle.   The concentration of dye in the urine rose in the next (4th) period to 260 per cent, a rate of .24 mg. per hour and increased in the 5th period to a figure of 500 per cent, 5½ times as concentrated as the perfusion fluid, a rate of .50 mg. per hour.

The rate of excretion of neutral red with tubular perfusion of the dye was therefore 8 times that obtained when the dye was administered to the glomeruli.   Summarizing twelve experiments shows that the average increase in the rate of excretion of the neutral red by tubules as contrasted to the glomerular output was thirty-five times, the range of variation being from eight to sixty fold.

If it be a fact, as the above experiments would seem to indicate that the excretion of the two dyes is by different mechanisms, phenol red being excreted chiefly by the glomerular apparatus and neutral red by the tubular system, then it should be possible to devise crucial experiments which will confirm indirectly the results described above that were obtained by direct observation.

If, for instance, the above hypothesis be true, it should be possible to administer both dyes to kidney at the same time in such a manner that no dye in any considerable amount appears in the urine.   The following experiment demonstrates that this can be done.

The perfusion experiments previously described were repeated, but to the glomerular circulation neutral red in a concentration of 12.5 mg. per 1000 cc. was supplied while a similar concentration of phenol red was led to the tubule capillary system.   Fig. 4 illustrates the results.   Although both perfusion bottles contained highly colored fluid, the urine in the ureteral canulae was practically colorless to the unaided eye.   The urine sample for each period was divided into separate portions, one being acidified to produce the red form of neutral red and another alkalinized to show the yellow form of phenol red.   In the first sample no phenol red could be demonstrated.   Neutral red in an amount averaging 15 per cent of the concentration in the perfusion fluid was present.

Clear Locke's solution, free of either dye, was now supplied to the tubular apparatus by the vein, and phenol red led to the glomeruli by the arteries.   In the next period (2) 570 per cent of the concentration in the perfusion fluid appeared

in the urine, a rate of 1.5 mg. per hour.   In the next period about one half as much
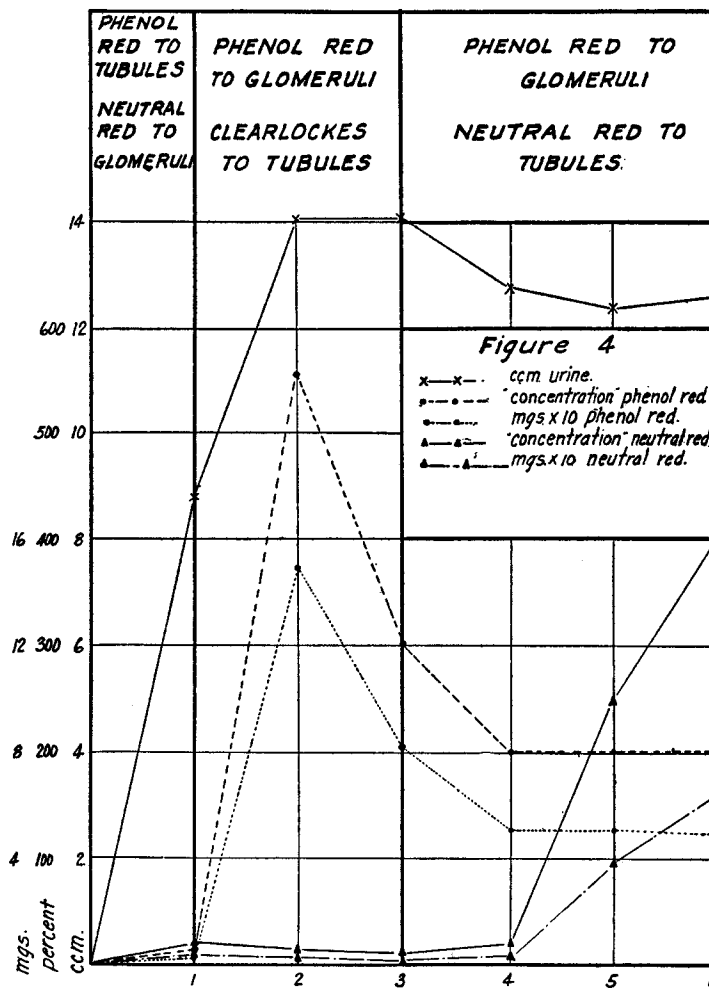was excreted.[1]

FIG. 4

Neutral red was now added in its original concentration to the fluid supplying
the tubular system, the phenol red still being present in the glomerular supply.

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[1] As we will show in a later study, the dye is excreted in greatest amount in
the first period and then falls to a fairly constant level.

By separating the urine as in the first periods, making one acid and the other alkaline, an approximate estimation of the amount of each dye could be made. Exact color matches in the colorimeter were impossible but rough estimations could be made. In the 5th period the two dyes were present in about equal concentration, 200 per cent for phenol red and 250 per cent for neutral red. In the 6th period the latter had increased to 400 per cent, the phenol red remaining approximately 200 per cent of the concentration existing in the perfusion fluid. The final rates of excretion for the two dyes was .5 mg. per hour for neutral red and .45 mg. per hour for phenol red.

It will be seen that the two dyes, when supplied to the kidney the "wrong way to" were not excreted in any significant amount. When the method of administration was corrected to correspond to the normal manner of excretion, both appeared in urine and were excreted at normal rates.

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If one of the dyes, neutral red, is excreted by the tubular apparatus, while the other, phenol red, is not excreted in any significant amount by these elements of the kidney but only by the glomeruli, then destroying the function of the tubules should affect the rates of excretion in a very different manner.

Such an experiment involves the complex question of abnormal activity of the tubule epithelium and is one which we shall discuss and study more fully in studies on experimental nephritis in the frog to be published later. Höber (2) has done a considerable amount of work on this problem in his studies of the physiological activity of the kidney and we can refer to his findings in applying the method to our immediate problem.

Höber has shown that when the tubule cells are anesthetized with urethane, their characteristic activities cease. There is no longer an absorption of water from the lumen of the tubule, hence a diuresis results. Sugar, which Wearn and Richards (5) have shown to be present in the glomerular filtrate, is also not absorbed from the urine as it passes down the tubule lumen, and hence appears in the urine, and the absorption of NaCl is also decreased. The urine, therefore, as a result of the abolition of tubular absorption, approaches in its constitution the character of the perfusion fluid.

In our experiment the rate of excretion of the two dyes was determined when such conditions had been produced by the action of urethane. Fig. 5 shows the result of such an experiment. Neutral red was supplied in the first 2 periods to

the glomeruli alone and, as in the previous experiments, was excreted through them at a very low rate, namely, .02 mg. per hour. The dye was then led to the tubular system and in three periods (3, 4, 5) an hourly rate of excretion 30 times as high as previously, or .60 mg. per hour, was attained. The urine during all these periods was normal in every way; the amount was not excessive, sugar was not present and its salt concentration was below that of the perfusion fluid. Urethane was
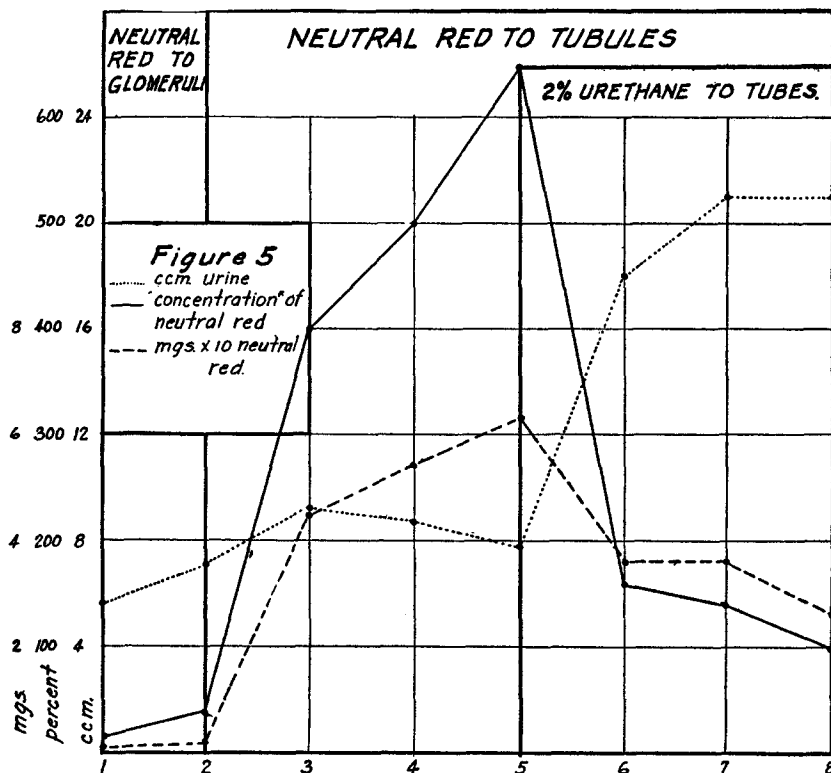

FIG. 5

now added in 2 per cent concentration to the perfusion fluid which was passing to the tubules. There was an immediate drop in the rate of excretion of the neutral red to about one-half its former figure (Period 6) and the rate continued to decrease during the next 2 periods, to a final figure of .22 mg. per hour, or one-third the value found under normal optimum conditions. All the evidence of tubular damage as described by Höber were present; there was a marked diuresis, sugar appeared in the urine and the salt concentration approached that of the perfusion fluid.

A similar experiment was now performed with phenol red. The results are shown in Fig. 6. The phenol red was introduced at once in this case into the glomerular circulation and as before was excreted at the relatively high rate of .3
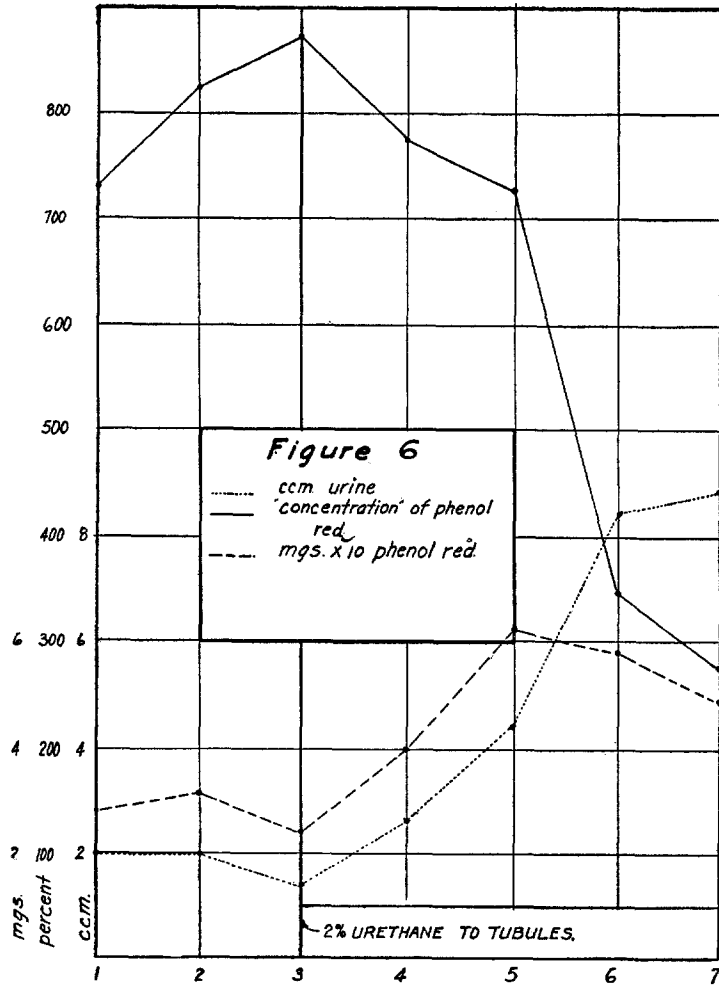

FIG. 6

mg. per hour. The urine contained no sugar, the salt concentration was only 40 per cent of that of the perfusion fluid and the amount of water was moderate. The same concentration of urethane, 2 per cent, was then introduced into the fluid which passed to the tubules. As the chart shows, in Period 6, sugar appeared in

the urine, the rate of water excretion increased 4 fold and the salt concentration rose, all evidences of tubular damage. The rate of excretion of phenol red, however, not only remained undepressed but actually increased.[2] The result is therefore the opposite of that obtained when neutral red was excreted, and gives further evidence which indicates the antithesis in the condition of excretion of the two dyes.

DISCUSSION

Since Nussbaum's original description of the double nature of the blood supply to the amphibian kidney and his experiments, based on this finding concerning the function of the tubules and glomeruli, numerous criticisms have appeared of the basic contention that the blood supply of the two systems is independent. One of the most recent of these is that of Smith (7) who from the result of certain experimental procedures comes to the conclusion that such rich anastomoses occur between the two systems that any interference with either the venous or the arterial systems will cause the unobstructed blood stream to supply both capillary systems. Perfusion experiments as done by many investigators, in so far as the renal portal vein is concerned do not therefore perfuse the kidney in the sense of supplying the tubule through a capillary bed.

A great deal of discussion would be necessary if the details of these experiments of Smith were to be adequately compared with experiments of other authors which are held by them to show the contrary results. For our purpose this can be avoided, for the good reason that our experiments are so designed and their results are of such a nature that in themselves they answer the objections of Smith and others. There is no doubt from our results that the excretion of the dyes is strikingly different when led to the kidney by the arterial and venous routes respectively, and that these differences are the converse of each other with the two dyes. In perfusion of phenol red through the renal portal system for example, the dye did not appear in significant amounts in the urine. This lack of excretion could not be, as Smith's objections might lead one to suppose, because it was passing through

[2] Hayman and Richards (6) have shown that the tubules absorb most dyes, including phenol red, so that abolition of the absorptive function of renal epithelium would increase the amount excreted under such conditions. A more detailed discussion of this point will be given in our studies on experimental nephritis.

the kidney directly to the post caval vein without passing through any capillary bed in the kidney, *because identical perfusion with neutral red through the same system was followed by the excretion of large amounts of dye.* And one can hardly suppose that this excretion of neutral red was the result of fluid reaching the glomeruli by anastomoses from the vein, for the direct introduction of the neutral red into the glomeruli was followed by the excretion of only a small amount.

Since Nussbaum's time there has been no question that anastomosis exists between the two systems. As Höber's experiments have shown (2) leakage from the glomerular system to the tubular is greater than the reverse flow from the venous capillaries to the glomeruli *under normal condition of relative pressures.* With abnormal relations of pressure in vein and artery this leakage of fluid and dye is increased, as our experiments with carbon particles and those of Smith and many others show. Furthermore, in the case of phenol red the apparent excretion of the dye, by the tubules, increases as this leakage increases. It may be true that this output of dye is due to the actual excretion of a small amount through the tubular system into which it is first introduced and that the dye is therefore excreted by both glomeruli and tubules as Tamura and his coworkers (8) have concluded from another type of experiment. But the increase in this apparent tubular excretion coexists with a demonstrable increase of leakage to the glomeruli, through which phenol red is readily excreted, and this fact leads us to believe that the excretion by the tubules is apparent rather than real. The converse we believe to be true of neutral red, the leakage which produces the apparent glomerular excretion of this dye being from the glomeruli to the tubules through the cells of which, as experiment shows, it is readily excreted.

In conclusion we wish to emphasize an important difference in the experiments described here from the majority of the previously reported perfusions of the frog kidney. The high arterial pressure as compared to that used by most investigators, combined with the size of our frogs, often 1000 grams in weight, has allowed us to obtain much larger samples of urine than those usually reported in such experiments. Their volume is, however, not abnormal, as Adolph (9) has shown that the average normal rate of water excretion by the frog when there is an excess of fluid available is 1.3 per cent of their weight per hour. This would mean that with our frogs a perfusion

which is normal in its production of water should produce from 7 to 13 cubic centimeters of urine per hour. No objection can be raised to the pressure in the glomerular circulation, which is high as compared to that which exists in the living frog, since it produces no abnormality in the urine. Repeated tests showed that the glomerular membrane was still intact, retaining gum arabic or albumin which had been added to the perfusion fluid, nor did the semi-colloidal neutral red pass through under this pressure, as our experiments have shown. All the other constituents of urine appeared in normal concentration and amount. Under the conditions of our experiments, therefore, the kidneys functioned in a normal manner, a statement which can not be made of experiments where volumes of urine of .19 cubic centimeter were excreted per hour. The added difficulty of accurate quantitative measurements under such conditions is also obvious.

CONCLUSIONS

1. Phenol red and neutral red are excreted by the perfused frog's kidney by different routes.

2. Phenol red is excreted chiefly through the glomerulus, neutral red through the tubules.

3. Some slight excretion of each of these dyes by the converse mechanism is possible, though there is no evidence in our experiments that necessitates such a conclusion.

4. The importance of methods leading to the production of a normal volume of urine by the perfused kidney is emphasized.

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