CCXXXIII. DIFFUSION EQUILIBRIA FOR THE ISOLATED FROG'S KIDNEY.

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THE isolated sartorius of the frog has been selected as particularly suitable for the study of the phenomena of muscular contraction owing to the quick diffusion of oxygen and metabolites across its substance, and the length of time it will therefore retain its normal activity. Here for similar reasons we shall consider the diffusion equilibria of the isolated frog's kidney (which in breadth and thickness resembles the sartorius muscle) with respect to substances introduced into a surrounding Locke's fluid. In the active isolated kidney of the frog without any flow of urine diffusion equilibria of substances in an external Locke's fluid are comparatively quickly attained. They are characteristic for the kidney and must be related to its special activity. The comparison is made with similar equilibria in kidneys under the influence of cyanide and hence inactive, and also with the sartorius muscle and other tissues.

The equilibrium concentrations of the inactive kidney will in general be lower than that of the surrounding fluid, since even with the freest permeability the amount of water available for solution is only about 80 % of the volume of the tissue. Where a substance under investigation is one which is normally concentrated in the urine we may expect, from the theory of active secretion, that its equilibrium concentration will exceed that of the inactive kidney.

There existed the possibility that the urine within the tubule of such kidneys would be so small in quantity that the difference between activity and non-activity in secretion could not be satisfactorily demonstrated in such a manner. The results have however shown unexpectedly large differences. For urea, phenol red and bromophenol blue (three typical "secreted" substances) not only do the equilibrium concentrations always exceed those for the inactive kidney but in many instances considerably exceed that of the substance in the surrounding Locke's fluid.

Where the substance under consideration, judging from the analysis of the frog's urine and blood, is diluted in the urine (*i.e.* is a re-absorbed substance), the equilibrium concentration in the active kidney may be expected to be less than in the inactive kidney. From a consideration of the behaviour of the mammalian kidney it was surprising to find that sodium sulphate was in general for the frog (*Rana temporaria*) a strongly re-absorbed substance, in the sense that the concentration of sulphate in the urine of the normal frog was found, in about 80–90 % of cases, to be only one-third or so of that of the plasma. In about 10–20 % of urines examined the relationship was the reverse, the concentration in the urine being about three to eight times that in the plasma. In a similar way diffusion equilibria for sulphate in the active isolated kidney

were in the great majority of cases (85 %) markedly less than those of the inactive kidney and for a small group markedly above.

The unexpected relations of sodium sulphate in the frog's kidney and the verification of certain principles believed to underlie renal secretion, already propounded elsewhere [Conway and Kane, 1929], led to a fuller investigation of this substance and to the collection of a large number of data on the sulphate equilibria of the kidney supplemented by observations on other tissues. These will be presented in this communication.

Methods.

Analytical procedure. Different methods for the analysis of minute quantities of sulphate have been recently described. Lorant [1929] converts the sulphate into hydrogen sulphide and estimates it as such; Van Slyke *et al.* [1927] determine it ultimately as a volume of nitrogen which is in relation to the original sulphate, and Hubbard [1927] precipitates as benzidine sulphate which is determined colorimetrically after oxidation with hydrogen peroxide and ferric chloride. The method selected at first was Wakefield's modification of Hubbard's method [1929]; later the benzidine sulphate precipitates were titrated in the manner described by Cope [1931]. Both colorimetric and titrimetric methods were found to give essentially similar results; the results to be described were however obtained (unless otherwise stated) by the titration method. The micro-burette used for the titration was not that of Rehberg [1925] but one described in a previous communication [Conway, 1934]. It is as accurate as the Rehberg burette and may be easily constructed in the laboratory. For the present determinations it contained 0.005 *N* CO₂-free alkali. The procedure adopted in analysing the kidneys for sulphate was as follows. The kidneys (usually two in number) were removed. They were then transferred to a weighed watch glass and rapidly weighed. After weighing they were transferred to a clean, dry, agate mortar with 1 ml. of distilled water and a little quartz sand (Merck's purest) and ground to a fine suspension in 2 minutes. (The quartz sand is in fact scarcely necessary as the kidneys are rapidly disintegrated in distilled water.) 1 ml. of 13.5 % trichloroacetic acid was now added and stirred with the mixture for another minute, and 2 ml. of distilled water were added and mixed. The fluid was subsequently transferred to a 15 ml. centrifuge-tube and centrifuged for about 5 minutes to clear the suspension. 3 ml. of the clear fluid were then taken, 10 ml. of 2 % benzidine in pure acetone added and the procedure was subsequently the same as that described by Cope. The final titration was carried out after adding 1 ml. of a weak aqueous solution of phenol red having the end-point colour. We found the phenol red more sensitive than phenolphthalein. Suitable blanks and controls were included in each series analysed. Since a knowledge of the sensitivity of the method is of importance in a consideration of the results, the following account may be given of the estimation of the sulphate in 0.1 ml. of a sodium sulphate solution containing 10 mg. S per 100 ml. This was delivered into centrifuge-tubes with the addition of water and trichloroacetic acid to bring to the usual trichloroacetic acid strength, the volume being 3 ml. before adding the benzidine reagent. Table I gives an account of the data obtained in the first five of a series of 24 such analyses. The standard deviation of the single determination was 0.6×10^{-3} mg. S, the 24 analyses being contained within the range -1.1×10^{-3} mg. and $+0.9 \times 10^{-3}$ mg. S. For the average of four or five analyses this error range will be halved corresponding approximately to ± 0.5 mg. S per 100 ml. for the analysis of 0.1 ml. deliveries. This is sufficiently accurate for our present purpose.

Table I.

No. of analysis	Burette reading for titration of benzidine ppt. cm.	Calculated quantity of sulphate mg. S
1	13.1	10.3×10^{-3}
2	12.7	10.0×10^{-3}
3	12.7	10.0×10^{-3}
4	11.5	9.0×10^{-3}
5	13.1	10.3×10^{-3}

Blank value = 0.25 cm. on burette; allowed for in the above readings.

The burette contained 0.005N KOH (CO_2 -free) and 1 cm. distance on it corresponds to 0.77×10^{-3} mg. S.

The analyses are the first five of a series of 24 on 0.1 ml. deliveries of a pure sodium sulphate solution containing 10 mg. S per 100 ml. (factor of 0.1 ml. pipette = 0.98).

Standard deviation for the total group of 24 is 0.6×10^{-3} mg. S.

Experimental procedure. The kidneys of the pithed frog were at once removed to filter-paper, trimmed of extraneous tissue and if not immediately used for analysis were placed in Locke's solution contained in a tube of about 5 ml. capacity. Oxygen saturated with water vapour was bubbled through rapidly so that the kidneys were constantly stirred, the oxygen being introduced through a fine tube fixed in position with a little sealing-wax. The Locke's fluid was renewed every 10 minutes for half an hour (this renewal was scarcely necessary as the volume of fluid was always sufficiently great—50 to 100 times the volume of the kidneys—to render the diffused material insignificantly small). After half an hour the kidneys were removed and if not immediately dried, weighed and analysed were removed to another similar tube holding some sulphate-Locke solution with or without potassium cyanide (0.02–0.04 %). The oxygen bubbling was then carried out as before, the kidneys being removed for analysis after the required interval.

In obtaining samples of blood for analysis the frog was pithed and a cannula quickly inserted into one carotid. Sufficient blood could be obtained as a rule in small tubes of 0.5 ml. capacity with pointed ends arranged on plasticine. The blood was allowed to clot, centrifuged and the serum taken for analysis. Urine was taken at the same time from the bladder if this contained any, the bladder being ligatured at both ends, removed and the urine taken.

Results.

The sulphate content of the serum, urine and kidneys of normal frogs (Rana temporaria). Table II gives the results of the analyses of blood and urine from

Table II.

No. of analysis	Serum-sulphate conc. mg. S per 100 ml.	Urine-sulphate conc. mg. S per 100 ml.	Urine conc. as % of plasma conc.
1	7.9	1.9	24
2	8.3	1.5	18
3	5.8	2.0	35
4	5.6	0.3	5
5	7.3	3.1	43
6	8.6	2.8	33
7	3.8	18.3	480

The above analyses were made in April, 0.1–0.4 ml. of serum or urine being used.

the same frog. Fig. 1 gives the distribution of the total analyses of sulphate in serum, kidney and urine. The mean concentration of sodium sulphate in the

frog's serum from 19 estimates is 6.2 mg. S per 100 ml. with a standard deviation of the mean of 0.4 mg. S per 100 ml. The distribution may be expressed by the standard deviation for the single analysis, namely 1.8 mg. S per 100 ml. (If we allowed for the distribution produced by the error of estimate this would show only a small reduction to 1.7 mg. S per 100 ml.) The results for the urine analyses fall into two groups, one containing the bulk of the observations and showing a marked absorption and a small group showing an equally marked secretion of sulphate. For the group of urine analyses (18 in number) fully carried out, four are much in excess of the usual blood figures. These urines are at once distinguished by the thick benzidine precipitates they cause and in

Fig. 1.

this way a number of samples were tested without proceeding fully with the analysis. It was found that rather less than 15 % fall in this group, the proportion varying with different consignments of frogs. For one consignment of 36 summer frogs no high concentration in the urine could be demonstrated. The average urine value for the absorbed group was 2.7 ± 0.4 S per 100 ml., the standard deviation of the individual determination being 1.5 mg. S per 100 ml.

The sulphate content of the normal kidney of the frog was found from 17 analyses to be 3.3 ± 0.3 mg. S per 100 g. or about half that of the plasma. The distribution of the analyses is shown in Fig. 1, the standard deviation of the individual determination being 1.1 mg. S per 100 g. In this figure for the renal sulphate is included 0.4 mg. S per 100 g. which is apparently indiffusible into the surrounding Locke's fluid and though estimated as sulphate, may not in fact be this substance or may have a merely analytical significance. This would leave 2.9 mg./100 g. S as the value of the diffusible inorganic sulphate in the kidney and so represent 47 % of that in the plasma. It will be seen below that about the same figure is reached for diffusion equilibria in sulphate-Locke which contains inorganic sulphate in the concentration of 10-100 mg. S per 100 ml.

The diffusion of sulphate from the isolated normal frog's kidney. The kidneys (usually two) were suspended in Locke's fluid free from sulphate and kept constantly stirred by bubbling oxygen through. The sulphate quickly leaves the kidneys so that in 2 minutes about 36 % of the initial amount has diffused.

In 30 minutes 89 % has diffused and, as previously indicated, further time allowed for the diffusion does not change this average figure.

Equilibrium concentrations of the kidney in cyanide-sulphate-Locke solution ("inactive" kidneys). With the cyanide-sulphate-Locke solution a sulphate equilibrium of 58 % (by weight) of the external fluid is reached in about 15 minutes and this figure is maintained very steadily up to 90 minutes, after which there is a comparatively sharp rise to a new equilibrium representing 74 % of the external sulphate. The experiments illustrated in Fig. 2 were made with a solution

Fig. 2. × Kidney. • Kidney (cyanide). + Liver. ○ Liver (cyanide). ▽ Sartorius. ◊ Sartorius (cyanide).

Fig. 3.

Table III.

External sulphate mg. S per 100 ml.	Active kidney		Inactive (cyanide) kidney	
	Sulphate conc. mg. S per 100 g.	Sulphate conc. as % of external sulphate	Sulphate conc. mg. S per 100 g.	Sulphate conc. as % of external sulphate
10	3.6	36 (4)	6.1	61 (4)
20	8.4	42 (4)	—	—
25	9.7	39 (4)	—	—
60	21.0	42 (9)	28.5	57 (9)
100	43.0	43 (4)	59.0	59 (2)

The figures in brackets represent the number of experiments.

The kidneys in all cases were first washed in Locke's fluid for 30 minutes, being stirred by a bubbling stream of oxygen. Subsequently the kidneys termed "active" were immersed in a sulphate-Locke fluid for 30 minutes and the "inactive" kidneys in a cyanide-sulphate-Locke fluid. The kidneys were in all cases stirred by a stream of oxygen.

containing sulphate in the strength of 50 mg. S per 100 ml. Varying the sulphate content of the external fluid (from 10 to 100 mg. S per 100 ml.) produces no change in the equilibria in the kidney when expressed as percentages of the external sulphate (see Table III). Fig. 3 shows the distribution of 12 individual analyses for the cyanide equilibria with external sulphate 20–50 mg. S per 100 ml. The mean of these cyanide values is 58, the standard deviation of the individual analyses being 3.7. (The figure shows also the distribution of values for the active kidney under similar conditions.)

Equilibrium concentrations of the kidney in sulphate-Locke solutions ("active" kidneys). Here as with the inactive kidneys the diffusion process tends to completion in about 15 minutes, though there is a subsequent slow rise which is obviously due to a shifting of the equilibrium value itself. From zero time onwards the sulphate content of the inactive kidney immersed in sulphate-Locke considerably exceeds that of the active kidney as may be seen from Fig. 2. The equilibrium values in Fig. 2 for active kidneys in sulphate-Locke were taken from the data obtained from a single large consignment of local frogs in June, the sulphate-Locke solution containing in all cases 50 mg. S per 100 ml. as sodium sulphate. The conditions were therefore uniform while the time of immersion was varied. (The results of previous experiments had shown an even lower figure for the equilibrium of the active kidneys of consignments earlier in the year.) From 5 to 30 minutes after immersion the increase of the sulphate concentration in the inactive (cyanide) kidney over that of the active amounts to 13–16 % of that of the external sulphate or 43–30 % of the actual sulphate concentration in the active kidney (see Table IV). As time proceeds the difference diminishes as may be seen from Fig. 2. The difference also appears at all concentrations (10–100 mg. S per 100 ml.) in the external sulphate-Locke, as shown in Table III.

Table IV.

Tissue	Period of immersion in sulphate-Locke mins.	Normal tissue		Tissue in cyanide		Increase in conc. of tissue over normal as % of external fluid
		Conc. as % of external sulphate. Mean value	No. of exps.	Conc. as % of external sulphate. Mean value	No. of exps.	
Frog's kidney	5	30	4	43	4	+ 13
	15	43	4	56	2	+ 13
	30	43	2	59	4	+ 16
	60	50	4	58	1	+ 8
	75	46	4	56	4	+ 10
Frog's muscle (sartorius)	2.5	10.7	4	9.0	4	- 1.7
	5	14.4	5	13.3	6	- 1.1
	10	17.1	4	15.5	2	- 1.6
	30	25.6	3	—	—	—
	60	24.6	3	24.8	6	+ 0.2
Frog's liver	5	20.4	3	16.1	2	- 4.3
	18.5	30.3	2	27.5	2	- 2.8
	60	27.6	3	23.0	2	- 4.6

Before immersion in sulphate-Locke or in sulphate-cyanide-Locke all tissues were washed in Locke for 30 minutes, being stirred by a stream of oxygen. The experiments were carried out at room temperature which varied from 20 to 25°. The experiments were performed in June and July.

When the external sulphate-Locke solution contains 10 mg. S per 100 ml., the equilibrium figure (after 30 minutes' immersion) appears lower than that for higher concentrations. With the colorimetric method this difference for the lower concentrations in the external fluid was even more marked.

It was previously noted that occasionally the urine-sulphate showed a much higher concentration than the plasma-sulphate, and that this occurred in about 15 % of the total number of samples examined. It might be expected that the "active" kidneys would also occasionally show evidence of a "positive" secretion of sulphate rather than a "negative" one.

This would appear in a higher equilibrium concentration in the active kidney than in the inactive, and this was found in a small proportion of the analyses. In 82 analyses (21 colorimetric) made on the active kidney 14 were markedly higher than for the inactive kidneys.

The distribution of the analyses for the active kidney is shown in Fig. 3, where the conditions are similar—30 minutes' immersion in the sulphate-Locke solution of kidneys from frogs of the same or similar consignments and 20–50 mg. S per 100 ml. in the external Locke fluid. Over this range of concentration it has been shown that no appreciable difference exists in the relative value of the equilibrium concentration. This value tends to a figure representing 41 % of the external sulphate, the mean value—considering only the kidneys which are “diluting” sulphate—is also 41 and the standard deviation of the single determination 4.4.

The proportion of times in which the high equilibria for the active kidney occurs is the same as that for the occurrence of high urine values.

Sulphate diffusion equilibria in muscle. The striking differences between the sulphate equilibria for the kidneys in cyanide-sulphate-Locke and in sulphate-Locke led naturally to a consideration of the possibility of similar differences in muscle and other tissues. The sartorius muscle of the frog was selected in the first instance. The dimensions in mm. of the average muscle investigated were $30 \times 3.5 \times 1.05$, and the average weight 115 mg. We may compare these with the average kidney dimensions for frogs received from abroad in March and April, which were in mm. $16 \times 3.0 \times 0.7$, and the average weight 35 mg. For local frogs received in May and June the average dimensions were $18.6 \times 3.5 \times 0.8$ mm. and the average weight 55 mg. It will be seen that the figures were somewhat similar for the breadth and thickness of the two tissues. The muscles were treated in a similar manner to the kidneys, receiving a preliminary washing in Locke's fluid for 30 minutes. Analysis of the muscles after this procedure gave an average value of 1.4 mg. S per 100 g. Since with the dimensions of the sartorius and the rate of entrance of sulphate practically all diffusible sulphate should have passed out in 15 minutes this apparent or fixed sulphate was deducted as a blank value. The muscles after this preliminary washing were then immersed in sulphate-Locke or cyanide-sulphate-Locke containing 50 mg. S per 100 ml. Oxygen was bubbled through as before and after varying times the muscles were removed for analysis in the same way as the kidneys. It was found that diffusion equilibria were reached in about the same time as with the kidneys, the diffusion process being nearly complete in 15 minutes. The equilibrium value for the muscle was much lower than for the kidney representing 25 % only of the volume of the muscle. The effect of cyanide was markedly different from that on the kidney. So far from effecting a much higher equilibrium concentration it caused a slight reduction. The results are shown in Fig. 2 and Table IV. The table shows a small reduction in the amount of sulphate entering the muscle due to the action of cyanide which is rather consistent over the whole period investigated (0–60 minutes).

Sulphate equilibria for liver sections. Sections were cut from the frog's liver having the average dimensions $11.2 \times 1.8 \times 1.1$ mm. These were treated in a similar manner to the other tissues. After washing for 30 minutes in Locke's fluid a blank value represented by 3.6 mg. S per 100 g. was found (average of four analyses ranging from 6.6 to 2.7 mg. S per 100 g.). The analysis of two unwashed sections gave 7.2 and 7.3 mg. S per 100 g. As before whatever significance this blank value may have it does not represent freely diffusible sulphate which would have all disappeared in the washing in Locke's fluid. The liver sections were then immersed for varying times in sulphate-Locke (50 mg. S per 100 ml.) in the manner previously described. The results are summarised in Table IV and Fig. 2. The equilibrium values are again practically reached in 15 minutes, the average value for the equilibrium in sulphate-Locke (50 mg. S

per 100 ml.) was 29 % of the external sulphate and 25 % for the sections in cyanide-sulphate-Locke, showing as with the muscle a small reduction due to the cyanide.

DISCUSSION.

It will be seen from the results that for about 85 % of frogs' kidneys (*Rana temporaria*) inorganic sulphate is—in the terminology of renal physiology—a strongly re-absorbed substance and in about 15 % just as markedly secreted. In this we suppose that all the urine volume comes from the glomerulus and corresponds there in composition to a blood-filtrate. In the one instance where a normal blood analysis and a corresponding highly concentrated urine were analysed the high urine concentration was not associated with a high but rather with a low plasma concentration. It is well known that the frog does not concentrate chloride at any plasma figure—so that there is no threshold value for secretion of chloride in the sense in which this is used for mammals, nor is it likely that a secretion threshold exists for sulphate but rather that the kidneys are concentrating or absorbing sulphate in reference to some special condition.

Corresponding to this relationship of urinary sulphate and plasma-sulphate we have the fact that the equilibrium concentration in the active kidney is, in the large majority of cases, considerably less than that of the inactive (or cyanide) kidney, the relationship being about 41 to 58, expressed as percentage of the sulphate in an external Locke-sulphate solution. The relationship is very clearly shown in the frequency distribution given in Fig. 3. The figure shows also that a small number of the equilibrium figures for the active kidney lie well above the region for the inactive (cyanide). These high values may be presumed to correspond to kidneys which "concentrate" rather than "dilute" sulphate.

These results are in accord with a view of renal secretion already propounded [Conway and Kane, 1929]. According to this view the main function of the glomerulus is to take control of the water secretion, the actual secretion and absorption of substances being a diffusion towards potential equilibria created by the active cells. The existence of such potential equilibria must be postulated even with the water absorption theory to explain the much more rapid passage relative to the water of substances such as chloride and glucose across the cells. This passage must inevitably occur towards the end of the absorbing region for urines which are low in chloride or glucose, and it may be emphasised that the passage is then from a low urine concentration to a relatively much higher blood concentration, so that the problem of special secretion is in reality contained in the theory which appears to avoid it, the direction of the process being merely reversed. The potential equilibria considered here are in one sense dynamic in that they necessarily involve the expenditure of energy in bringing a substance such as sulphate from a lower urinary concentration to a higher blood concentration. If a flow of blood were maintained so that there was insufficient time for it to reach equilibrium, then sulphate would pass continuously across the active cells from the flowing urine into the blood-stream. If the blood were stationary, and a sufficient oxygen supply conceivable, the final blood concentration would be higher than that in the cells or in the urine. This condition is simulated by immersing the kidney in an oxygenated sulphate-Locke solution. It will be seen that this implies that for a sulphate-Locke solution of constant composition in which the kidney is immersed the concentration of sulphate in the active organ is less than in the inactive. This is the experimental result obtained and described above.

It will be observed that this explanation need involve no other cardinal assumption than that a glomerular fluid is formed similar in composition to a blood-filtrate. It does not state how the potential equilibria of the active cells are brought about, it merely considers their necessity. It involves the consequence that for secreted substances the opposite result should be obtained, namely that the total concentration of the active kidney at equilibrium should be higher than that of the inactive kidney. As before mentioned this difference has been fully demonstrated in this laboratory for urea, phenol red and bromophenol blue.

SUMMARY.

1. The average concentration of sulphate in the blood-plasma of 19 normal frogs (*Rana temporaria*) was found to be 6.2 expressed as mg. S per 100 ml. The distribution is given by the standard deviation—1.8 mg. S per 100 ml.

2. The urine concentration is in general much lower than that of the blood, approximating to one-third or less of its value. A small group showed the urinary sulphate to be concentrated like urea.

3. The concentration of sulphate in the kidney is likewise much less than in the blood, tending to about 40 % of its value.

4. The sulphate passes freely in and out of the frog's kidney in relation to the concentration of sulphate in a surrounding sulphate-Locke fluid. Equilibrium is practically reached in 15 minutes. For the active kidney the diffusion equilibrium tends to a value of 41 % of that of the external sulphate. For the inactive kidney (cyanide) the equilibrium concentration is 58 % of that of the external sulphate (or 72 % of the total possible concentration with full and equal permeation).

5. The equilibrium concentration in muscle is 25 % by volume of the external sulphate and for liver sections 29 %. Addition of cyanide causes a slight reduction in these figures in marked contrast to its effect on the kidney.

6. The rates at which the equilibrium figure is reached by diffusion are the same for muscle, liver, active kidney or inactive kidney although the equilibrium concentrations differ considerably.

7. The results are in accord with a diffusion-secretion theory of the kidney propounded elsewhere.

REFERENCES.

- Conway (1934). *Biochem. J.* **28**, 283.
— and Kane (1929). *Amer. J. Physiol.* **88**, 1.
Cope (1931). *Biochem. J.* **25**, 933.
Hubbard (1927). *J. Biol. Chem.* **81**, 713.
Lorant (1929). *Z. physiol. Chem.* **185**, 245.
Rehberg (1925). *Biochem. J.* **19**, 270.
Wakefield (1929). *J. Biol. Chem.* **81**, 713.
Van Slyke, Hiller and Berthelsen (1927). *J. Biol. Chem.* **74**, 659.