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

II. UREA.

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In a previous communication [Conway and Kane, 1934] it was shown that urea existed in the normal frog's kidney considerably in excess of that in the blood or of the sartorius muscle. This latter was found to have a urea concentration which might be expected to arise from a passive equilibration with the blood, the urea diffusing into all the water of the tissue.

It was also shown that the urea in the normal frog's kidney diffused outwards at the same rate as it diffuses into or out of muscle or into the inactive kidney. The diffusion coefficient of urea was found to be the same for the kidney as for muscle within the error limits of such determinations, its value being $1{\cdot}1 \times 10^{-5}$ (cm.^{2}/min.).

In the present investigation the point of chief interest was to discover the urea diffusion equilibria for active and inactive kidneys immersed in urea solutions. It will be shown that urea passes from urea-Locke solutions into active kidneys having a lower urea concentration until the total concentration of the tissue far exceeds that of the external fluid. When the tissue is rendered inactive by cyanide the urea enters only to the extent that may be expected for a passive equilibration with the tissue water.

In this process a freely soluble substance is being concentrated by the active kidney and the form in which it is concentrated appears to leave it in free solution and in such a condition that it can diffuse outwards at the same rate as urea from the inactive kidney or from muscle.

The facts of this urea diffusion are of such importance for renal theory that the method used for the analyses should be very carefully certified to give accurate data for the true urea content. There is all the more reason for this as the urease ferment extracts as ordinarily prepared may react to produce ammonia with the frog's kidney in addition to the urea ammonia and the free ammonia which is formed in all such surviving tissues.

It will be shown that bringing the kidney suspension to the boiling point in a small tube, and then immersing this in boiling water for five minutes completely eliminates the production of "extra" ammonia (*i.e.* other than urea ammonia) by the urease ferment. The procedure also stops any further production of free ammonia in the tissue suspension itself.

The results so obtained with the urease method used on boiled tissue suspension have been compared with xanthydrol determinations in a sufficient number of analyses to show that both methods give identical results.

The nature of the "extra" ammonia formed by urease ferment extracts on fresh unboiled renal suspension is in itself of much interest, and though not concerning us particularly in this communication, some of the main conditions of

its formation will be necessarily demonstrated in a critical analysis of the urease method.

As a result of this investigation of method it might be inferred that the results given in the previous communication were a little too high. The error would not exceed 7 mg. per 100 mg. kidney, and this amount does not invalidate any of the conclusions there drawn. It necessitated a further investigation of the diffusion coefficient. We expected to find this somewhat higher than in the last determination—namely about $1{\cdot}2 \times 10^{-5}$, instead of $1{\cdot}1 \times 10^{-5}$. However, the repeated determinations in November and December gave again the same figure (in fact very slightly less). We may explain this by supposing that the "extra" ammonia in the earlier experiments was, as previously considered, entirely negligible.

Methods.

(*a*) *Urease method.*

The method used has been described in detail in previous communications [Conway, 1933; Conway and Kane, 1934]. In the present determinations a weighed amount of tissue was ground with a measured volume of water and a little pure quartz sand (Merck) in an agate mortar. The quantities used were such that 0·5 ml. of the centrifuged ground mixture contained as a rule about 5–20 mg. tissue. The fluid was decanted after centrifuging to another rather narrow tube, and after the level had been marked was brought momentarily to the boiling point. The tube was then immersed in a beaker of boiling water for five minutes, being covered with a watch glass. Any fluid loss was made up after cooling—the loss being generally negligible.

0·5 ml., as an aliquot part of the fluid, was transferred to the outer chamber of an absorbing "unit", the inner chamber containing 1 ml. 0·001 *N* sulphuric acid (made up with 20 % alcohol and containing methylene blue and methyl red [Conway and Byrne, 1933]. The remaining procedure has been already described in detail. The titrations at the end were carried out with 0·005 *N* carbon dioxide-free alkali from the horizontal micro-burette previously described [Conway, 1934].

The accuracy of the method. It has been already shown [Conway, 1933] that the degree of hydrolysis of the urea by the urease ferment, and the absorption of the formed ammonia proceed to 99 % at least of the theoretical. The relative absorption rate for ammonia quantities of say 10 γ ammonia-nitrogen is the same as for 10,000γ. The variable error of ammonia determinations down to quantities of 10γ ammonia-nitrogen was approximately 0·5 % expressed as a coefficient of variation. The determination of urea quantities of the order of 40γ urea-nitrogen had a coefficient of variation of 1·2 %. The slight difference may be attributed in part to the use of 1 ml. of fluid in one procedure and only 0·2 ml. in the latter. No very appreciable variable error is therefore introduced by the hydrolysis of the urea itself.

In the present investigation we are concerned with the analyses of urea or ammonia quantities of the order of 3γ of nitrogen or less. From an examination of the analyses of the ammonia content of the stock urease extract an excellent opportunity presents itself of examining the variable error in the routine determination of urea- or ammonia-nitrogen of about 1γ. In four experiments following on one another 20 such determinations were carried out. The average nitrogen analysed was 0·65γ. Eighteen of the results did not deviate from this figure by more than 0·1γ, the standard deviation of the series being 0·06γ. It is possible to reduce the error further by slower titrations and stricter attention to

the practically complete absence of carbon dioxide from the titrating alkali. Without this refinement however the accuracy attained is quite sufficient for the present investigation and represents no more than 3–5 % error in the analysis of 3 γ of ammonia- or urea-nitrogen.

We doubt if any other method could compare with this for combined accuracy, simplicity and ease of working with such minute quantities of ammonia. The present and the previous communication are based on over 1000 such analyses.

The question of the "extra" ammonia produced by the urease extract. The various ammonia fractions (other than the reagent ammonia) that arise in a urease analysis of fresh kidney suspension may be listed as follows:

(1) Ammonia from the urea in the frog's kidney.

(2) Ammonia from such precursors as adenylic acid and formed in all such surviving tissues, the amount increasing with the time after removal.

(3) Ammonia formed from an interaction between some substance in jack bean extract interacting with some substance other than urea in the kidney.

To investigate the amounts and changes of these quantities during the course of extended incubation the following experiments were carried out, the volume and reaction of the analysed mixture being in each case the same. Groups of units were set up for the addition of 1 ml. of saturated potassium carbonate after various incubation times (conducted at 37°). These units contained the following mixtures in the outer chamber:

I. 0·5 ml. neutral phosphate (0·05 *M*).
0·5 ml. of centrifuged kidney suspension (containing about 5–20 mg. per ml.).
0·5 ml. glycerol (diluted 1 in 20).
0·5 ml. water.

I *a*. Same as I except that the centrifuged suspension was boiled in the manner already described.

II. 0·5 ml. of glycerol-urease extract (diluted 1 in 20).
0·5 ml. centrifuged kidney suspension.
0·5 ml. neutral phosphate (0·05 *M*).
0·5 ml. water.

II *a*. Same as II except that boiled centrifuged kidney suspension was used.

Similar units were set up containing the phosphate, glycerol and urease in the same volume of fluid, and also containing merely the phosphate and glycerol in order to ascertain the reagent ammonia.

Subtracting the reagent ammonia in each case, the ammonia from I and I *a* gives the free ammonia in the unboiled kidney suspension and the boiled respectively. Subtracting these values as well as the reagent ammonia from II and II *a* respectively one obtains the ammonia produced by the interaction of the urease ferment extract with the unboiled and boiled kidney suspensions.

Fig. 1 shows the mean results obtained in five experimental series, in each of which the incubation periods (before the addition of saturated carbonate) were maintained from 15 minutes to 3 hours.

A number of conclusions may be drawn from these results.

(1) After 15 minutes' incubation with urease all further ammonia formation stops in the boiled kidney suspension. The ammonia so formed (the original free ammonia being allowed for in Fig. 1) represents beyond any doubt the true urea content of the tissue and is identical with that found in xanthydrol determinations.

(2) The ammonia formed by the action of the urease extract on the fresh kidney suspension shows a linear increase up to 90 minutes' incubation and then remains steady up to 180 minutes. Extrapolation to zero time meets the line for the boiled suspension.

(3) The free ammonia of the boiled suspension also does not alter with incubation.

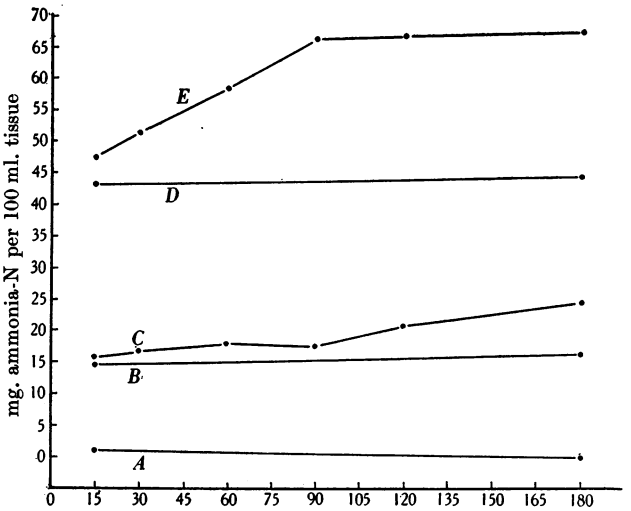

Fig. 1. The experiments from which the above figure is drawn are described in the text. The points are the means obtained in five experimental sets. *A*, Ammonia-N formed by interaction of boiled urease and kidney suspension; *B*, free ammonia-N in boiled suspension; *C*, free ammonia-N in unboiled suspension; *D*, ammonia-N formed by interaction of urease and boiled kidney suspension; *E*, ammonia-N formed by interaction of urease and unboiled kidney suspension.

(4) The free ammonia of the unboiled suspension rises slightly as the incubation time increases to 90 minutes and then begins to rise steeply. Clearly some marked change occurs around this period, with unboiled suspension under the above conditions. (The further precise elucidation of the "extra" ammonia formation by the interaction of the fresh kidney suspension and the urease extract does not concern us here. As a working hypothesis we may suppose however that a ferment such as arginase or a deaminase exists also in the urease extract and acts on arginine or a similar substance produced by proteolysis in the incubated tissue. Some further facts may be mentioned in support of this hypothesis. Doubling the concentration of the urease doubles the rate of formation of this "extra" ammonia. Doubling the concentration of the renal suspension causes the same result. Boiling the urease extract eliminates any ammonia formation due to the interaction of the extract and the suspension.)

(*b*) *Xanthydrol method.*

The xanthydrol method was also used in a certain number of analyses of kidney suspensions, diffusates and blood from the same frogs, to confirm further the findings of the urease method. In using this method 4 ml. of the renal suspension or diffusate containing approximately 1 mg. urea per 100 ml. were measured into a 15 ml. centrifuge-tube, and 1 ml. of the Tanret-Polonowski

reagent was added (tungstic acid precipitation was used for the first experiment). The tube was centrifuged for 15 minutes, 4 ml. of the clear fluid being taken in another 15 ml. centrifuge-tube and 4 ml. of a xanthydrol mixture (freshly formed by the addition of 5 parts of 5 % xanthydrol in methyl alcohol to 3 parts of glacial acetic acid) added, the fluids being mixed with a thin glass rod. The mixture was allowed to stand overnight. It was then centrifuged for 15 minutes. The fluid was very carefully decanted and the tube allowed to drain upright for a few minutes on absorbing filter-paper. 2 ml. methyl alcohol saturated with dixanthydryl urea were then added to the tube and the solid residue was stirred up. The tube was again centrifuged and the procedure repeated. After the final centrifuging the draining was allowed to continue for 5 minutes and then the end of the tube wiped with filter-paper. Up to this point the method used was largely similar to that used by Rehberg *et al.* [1932]. The further procedure consisted in adding 2–5 ml. of 50 % sulphuric acid and stirring with a glass rod. This is an adaptation of the method used by Beattie [1928].

After some time the yellow colour produced was examined in a micro-colorimeter, using a standard formed by dealing in an exactly similar manner with 4 ml. of a 1 mg. per 100 ml. urea solution instead of the kidney suspension. Suitable blanks were also carried out. Duplicate analyses were made in each case and colorimeter readings taken by independent observers.

The normal relation between the urea concentrations in the blood and kidney of the frog (Rana temporaria).

The relation found in the spring frogs has a mean value of 45 mg. per 100 ml. blood to 85 mg. per 100 ml. in the kidney. A new series of 14 analyses, with the revised technique, on boiled kidney suspensions gave an average of 82 mg. per 100 ml. The corresponding blood values (for 10 experiments) were higher for these months (November to February) than in summer, giving an average value of 56 mg. urea per 100 ml. The individual results are given in Table I. For the blood analyses 0·1 ml. was taken from each frog and mixed, the analysis being subsequently carried out on the mixed blood.

In a series of seven experiments not only was the urea in the boiled kidney suspensions and corresponding blood samples determined, but also the amount that diffused freely outwards in two hours from the companion control kidneys. These were immersed in about 50 times their volume of Locke solution and stirred by a stream of oxygen. The average value for urea in the kidneys of this series was found to be 85 mg. per 100 ml. The analyses of the diffusate showed that on the average 75 mg. urea per 100 ml. kidney diffused out in two hours into the Locke solution. One set (No. 5 in Table I) shows an aberrant figure for the renal urea in relation to the diffused quantities. Omitting this as probably erroneous the average values are 83 mg. urea per 100 ml. for the kidney and 76 mg. urea diffused per 100 ml. The average blood value in these six sets was 55 mg. per 100 ml. An excess of 40 % over the blood urea value has therefore diffused freely from the kidneys in two hours.

An average value of 1·3 mg. urea per 100 ml. was present in the Locke solution at the end of the diffusion period and this we may presume to be in equilibrium with about 2 mg. per 100 ml. of kidney tissue. The total diffusing concentration was therefore 81 mg. urea per 100 ml., so that 94 % of the total possible has diffused in two hours (practically the same figure is maintained if we include two extra sets for which the blood analyses are not available, the average percentage diffused is then in fact 95.

Table I.

No. of exp.	No. of frogs	Av. wt. of single kidney mg.	Kidney (boiled suspension) mg. urea per 100 ml.	mg. urea diffused per 100 ml. in 2 hrs. Calctd. from diffusate	mg. urea diffused per 100 ml. calc. from boiled diffusate (2 hrs.)	Kidney after diffusion (boiled suspension) mg. urea per 100 ml.	Blood mg. urea per 100 ml.	Blood (boiled) mg. urea per 100 ml.	Kidney diffusate mg. urea per 100 ml.
1	3	40	66	59	58	8	30	—	1·1
2	3	35	125	107	—	5	86	—	1·9
3	3	43	77	72	75	—	62	—	1·4
4	3	38	85	87	92	—	68	68	1·6
5	3	36	105	70 (?)	73	—	54	54	1·2
6	4	25	82	75	81	—	—	—	1·2
7	2	53	70	59	59	—	—	—	—
8	5	40	70	61	—	—	36	—	1·2
9	4	41	65	64	—	—	48	—	1·0
10	3	30	79	—	—	—	—	—	—
11	4	25	82	—	—	—	—	—	—
12	3	22	66	—	—	—	—	—	—
13	2	65	93	—	—	—	—	—	—
14	2	71	79	—	—	—	—	—	—

The amount of tissue analysed ranged from 7 to 13 mg. in 0·5 ml. of suspension. Where diffusions (2 hours' duration in Locke, stirred with oxygen) were carried out, one kidney from each frog was taken and the group transferred to Locke, the companion tissues being ground to a suspension in the usual way and analysed. For further details see text.

The average weight of the kidneys examined was 40 mg. and the theoretical amount that would diffuse in two hours—the diffusion coefficient being $1{\cdot}1 \times 10^{-5}$ is 98 %. The difference between the actual and the theoretical is therefore insignificant, and, as shown in section III, after three hours' diffusion 97 % is recoverable from the Locke fluid.

Table II.

No. of frogs	Av. wt. of single kidney mg.	Urea conc. of kidney mg. per 100 ml.		Urea diffused in 2 hrs. mg. per 100 ml. of kidney		Urea conc. of blood mg. per 100 ml.	
		Urease	Xanthydrol	Urease	Xanthydrol	Urease	Xanthydrol
6	51	—	104	—	101	—	72
5	40	70	69	61	59	36	36
4	41	65	66	64	61	48	49

One kidney from each frog was grouped and a suspension made in 12 ml. water; 4 ml. quantities were taken for the xanthydrol analyses, 0·5 ml. quantities were taken for the urease analyses. For the analysis of the diffused quantities the companion tissues were immersed in 12 ml. of Locke and stirred with a stream of oxygen for 2 hours, the Locke fluid being subsequently analysed. 0·1 ml. blood was taken from each frog and the mixed blood analysed.

The first experiment with the kidneys from 6 frogs was carried out in December, the remaining experiments in February from fresh consignments.

The ratio between the blood urea and the renal urea in the summer frogs was found to be 1 : 1·89, the ratio for the winter frogs 1 : 1·49, and as we have seen, nearly the entire amount of the renal urea freely diffuses outwards. Three sets of experiments in which the xanthydrol method was used with duplicate analyses, gave a ratio of blood to kidney urea of 1 : 1·43 for winter frogs. The average urea content of the kidney was 80 mg. per 100 ml. and the average diffused quantity into a large volume of Locke 74 mg. urea per 100 ml. tissue. Using the same considerations as above this means that 95 % of the total possible has diffused, the theoretical quantity being again 98 %. Table II contains the details of these

experiments and shows that in two sets where series of corresponding analyses were made with the urease method, practically identical results were obtained.

The xanthydrol results are therefore in complete agreement with the urease figures.

Diffusion relations of urea for the isolated frog's kidney.

(*a*) *The diffusion coefficient.* It was considered advisable to make a repeated determination of this coefficient with the revised technique. In the method adopted 12 kidneys were immersed in 20 ml. cyanide-Locke (cyanide concentration—0·02 % KCN) and kept stirred with a stream of oxygen saturated with water vapour.

0·5 ml. was removed at varying intervals ranging from 5 to 180 minutes. Over this period about three additional quantities of 0·5 ml. were removed to determine the free ammonia content. The whole course of the diffusion could be examined from the one group of kidneys without the need of comparing with the companion tissues for each determination after a given time period. The results are given in Table III. The mean value of the diffusion coefficient up to a period when 70 % was diffused was found to be $1{\cdot}09 \times 10^{-5}$, showing therefore no change from the previous determination and indicating that the analyses summarised in the previous paper, from which the diffusion coefficient was determined, were inappreciably different from the true figure. Table III shows also that the whole course of the diffusion is very close to the theoretical curve based on a coefficient of $1{\cdot}095 \times 10^{-5}$ and that after three hours' diffusion 97 % of the theoretical is recoverable.

Table III.

Diffusion period mins.	Amount diffused per 100 ml. kidney mg.	Amount diffused calculated from diff. coeff. of $1{\cdot}095 \times 10^{-5}$ mg. per 100 ml. kidney	Diffusion coeff. calculated from the diffused quantities (cm.²/mins.)
5	25·2	23·6	$1{\cdot}26 \times 10^{-5}$
10	32·9	33·7	$1{\cdot}03 \times 10^{-5}$
20	45·3	48·0	$0{\cdot}99 \times 10^{-5}$
30	57·8	57·1	$1{\cdot}10 \times 10^{-5}$
60	71·0	73·3	—
180	78·0	81·3	—

Temperature, 17°.

In the above experiment 12 kidneys from six frogs of one consignment and weighing 533 mg. were introduced into 20 ml. Locke's fluid and stirred with oxygen. 0·5 ml. quantities were removed at definite times for the urea analysis, three extra quantities of 0·5 ml. being taken over the three hours' period of the immersion for determination of the ammonia content. The kidneys at the end were also analysed, being ground up in 3 ml. of water.

The urea content of the kidneys at the beginning of the immersion was calculated from the analyses as 84·3 mg. per 100 ml. At the end the Locke fluid had a concentration of 2·25 mg. urea per 100 ml., the diffusing concentration therefore being approximately 82 mg.

The slight difference at the end of the experiment between the actual and theoretical quantities diffused may be attributed to the swelling of the kidneys.

(*b*) *Urea diffusion equilibria for the isolated kidney.* Before immersing the isolated kidneys in urea-Locke solutions they were previously washed in ordinary Locke for 20–30 minutes. In this way the concentration of urea was reduced from approximately 80 mg. to about 30 mg. per 100 ml. One of each pair of kidneys were then grouped together and analysed, the companion tissues being immersed and stirred with oxygen in urea-Locke and urea-cyanide-Locke solution respectively. In this re-immersion—if we consider the diffusion coefficient of $1{\cdot}1 \times 10^{-5}$—it would take over two hours before a practically complete equilibrium would occur; after this time there appeared some danger of the activity of

the kidney being much reduced. For this reason the time of re-immersion in the urea-Locke was confined to 30–45 minutes, the movement of the urea being studied over this period. The results of this study are sufficient and conclusive in establishing the main point of interest—namely, the concentration of urea by the active kidney from an external fluid. That the time was insufficient for establishing full equilibrium only lends emphasis to the results. At the same time it may be pointed out that about 70–80 % of the total movement of the urea towards equilibrium will have occurred and that from a consideration of the diffusion coefficient the equilibrium figure may be calculated.

A large number of experiments of this type were collected before the revised technique was set going. The individual results were obtained from the one pair of kidneys, one kidney being analysed after the 30-minute washing period and the other immersed for a further 30 minutes in urea-Locke (or urea-cyanide-Locke), the kidneys being stirred in all cases with a stream of oxygen. The concentration of the urea in the external Locke was either 20, 40 or 80 mg. per 100 ml. The results are summarised in Table IV.

Table IV.

		Inactive kidneys (cyanide)			Active kidneys			
Conc. of urea in the urea-Locke mg. per 100 ml.	Av. wt. of single kidney mg.	No. of exps.	Mean conc. before immersion in urea-Locke mg. urea per 100 ml.	Mean conc. after immersion in urea-Locke mg. urea per 100 ml.	No. of exps.	Mean conc. before immersion in urea-Locke mg. urea per 100 ml.	Mean conc. after immersion in urea-Locke mg. urea per 100 ml.	Increase in mean conc. of active over inactive kidneys mg. urea per 100 ml.
20	40	4	27	24 (21–26)	6	31	41 (27–71)	+17
40	33	6	31	37 (30–48)	14	33	50 (31–103)	+13
80	45	5	29	56 (51–60)	17	26	72 (42–104)	+16

In each experiment one kidney from one frog was analysed after the washing period of 30 minutes in Locke, and the companion kidney after the immersion period in urea-Locke.

The mean value of the urea content of the kidneys after the 30-minute washing is in all the groups within the range 30 ± 4 mg. per 100 ml. After re-immersion for 30 minutes in Locke containing 20, 40 or 80 mg. urea per 100 ml. the mean values for the inactive (cyanide) kidneys were 24, 37 and 56 mg. urea per 100 ml. respectively. The mean values for the active kidneys re-immersed in Locke containing the same urea concentration, but without cyanide, were 46, 50 and 72 mg. per 100 ml. In each group, therefore, a large excess of urea has diffused into the active kidney in comparison with the inactive kidney. It will be seen from the table that after re-immersion in urea-cyanide-Locke containing 20 mg. per 100 ml. urea the value falls over the period from a mean value of 27 mg. urea per 100 ml. to a mean value of 24 mg., whereas the urea content for the active kidneys rises from 31 to 46 mg. urea per 100 ml.

After immersion in the 80 mg. per 100 ml. urea solution, the content of the active kidney increases on the average from 26 to 72 mg. per 100 ml. There is here insufficient time for the value of the kidney to exceed that of the external fluid, but it exceeds the corresponding value for the inactive kidney by 16 mg. urea per 100 ml.

The question arises—does the movement of urea in the inactive kidneys indicate a passage towards a mere passive equilibrium? Considering the average weights of the kidneys examined in the three groups of cyanide-urea immersions, namely, 40, 33 and 45 mg., it may be calculated from the diffusion coefficient ($1{\cdot}1 \times 10^{-5}$) that in 30 minutes 72, 76 and 70 % of the equilibrium amount will

have diffused. From these figures and those in Table IV the equilibrium values for the three groups of cyanide immersions will be 23, 39 and 68 mg. per 100 ml. (the values after 30 minutes being 24, 37 and 56). From a mere passive equilibrium we should expect 17, 34 and 67 mg. urea per 100 ml. The experimental values exceed the theoretical therefore by +6, +5 and +1 mg. urea per 100 ml. or an average of +4 mg. per 100 ml.

Apart from the fact that this quantity is in itself small, it is probably due to an analytical error arising from the "extra" ammonia formed after 15 minutes' action of the urease extract on the kidney suspensions.

Applying the same calculations to the three groups of active kidneys, the equilibrium values are 51, 55 and 95 (the 30-minute values being 46, 50 and 72). These values exceed those for a passive equilibrium (17, 34 and 67) by +34, +21 and +28 mg. urea per 100 ml. They are of about the same order as the difference in urea concentration between the normal frog's kidney and that of the blood, namely 40 mg. per 100 ml. found for the summer frogs and 27 per 100 ml. for the winter frogs.

In the above analyses we were concerned with individual tissues the average values of which are summarised. The individual results, particularly of the active kidneys, gave, as may be expected, rather wide variations. A further group of five experiments was carried out with the revised technique, groups of three kidneys being used for each analysis. The conditions in these experiments were made as uniform as possible in comparing the active with the inactive kidneys. In each experiment of this group 6 pairs of kidneys were taken from six frogs of the same consignment and selected for their similarity in size. The 6 pairs were washed in Locke, being stirred with oxygen for 20 minutes. Three of the pairs were divided (all the pairs were held together by a small amount of uncut tissue) and three of the kidneys taken for analyses, the remaining three being immersed in Locke containing 40 mg. urea per 100 ml. The three remaining pairs were treated similarly, the companion tissues being immersed in cyanide-Locke containing also 40 mg. urea per 100 ml. The kidneys were immersed in the respective urea solution for 45 minutes, being subsequently analysed.

Table V.

			Inactive kidneys (cyanide)		Active kidneys		
No. of exp.	Av. wt. of single kidney mg.	Temp.	Conc. of urea in 3 kidneys before immersion in urea-Locke mg. per 100 ml.	Conc. of urea in companion tissues after immersion in urea-Locke mg. per 100 ml.	Conc. of urea in 3 kidneys before immersion in urea-Locke mg. per 100 ml.	Conc. of urea in companion tissues after immersion in urea-Locke mg. per 100 ml.	Increase in mean conc. of active over inactive kidneys mg. urea per 100 ml.
1	31	12	32	33	32	42	+ 9
2	31	17	27	42	30	51	+ 9
3	44	18	32	35	30	47	+12
4	39	19	37	34	39	57	+23
5	35	19	33	34	40	59	+25

The experiments are described in the text. In the table the results are arranged in order of the room temperatures at which the experiments were conducted.

Each analysis in the above table has been determined from a group of three kidneys.

The results are shown in Table V and completely confirm the previous results. After the 20 minutes' washing the average value of the companion tissues to those immersed subsequently in the cyanide-urea-Locke is 32·2 mg. urea per 100 ml. After immersion the average is 35·6. The average weight of the kidneys is 36 mg. After 45 minutes with a diffusion coefficient of $1{\cdot}1 \times 10^{-5}$, 86 % will have diffused, so that the final equilibrium value should be 36·2—very slightly above the 45-minute figure. The amount for a purely passive equilibrium is

34 mg. urea per 100 ml. This is in fact the figure reached in four out of five of the experiments, the remaining figure of 42 mg. per 100 ml. being somewhat aberrant.

Reviewing now the active kidneys of this series, in each experimental group the value is much higher than for the inactive kidneys, the difference ranging from 9 to 25 mg. urea per 100 ml. with a mean value of 16 mg. It may be noted that the lower figures of the group are associated with low external temperatures.

Prior to immersion in urea-Locke the average value for the companion (control) tissues of the active kidneys is 34 mg. urea per 100 ml., the average after immersion being 51 mg. urea per 100 ml.

Applying the same calculation as above, with the cyanide kidneys the equilibrium value appears as 54 mg. urea per 100 ml. or 18 mg. in excess of the 36 mg. found with the cyanide kidneys.

Discussion.

Here we may emphasise the fact that the concentration of urea by the active isolated frog's kidney from an external fluid has been definitely proved, the formation of a glomerular fluid by filtration during this process of concentration being an impossibility.

The proof depends on a large number of experiments, and selecting one group of these for illustration, it is briefly as follows. The kidneys of a number of frogs were removed and washed in Locke's fluid for 20 minutes, being stirred—as in all the immersions described here—by a stream of oxygen. Their concentration was thereby reduced to 34 mg. urea per 100 ml. Some were now immersed in Locke containing 40 mg. per 100 ml. of urea, and others in a similar urea-Locke containing cyanide, the immersion being continued for 45 minutes. The resulting concentration of the inactive (cyanide) kidneys was 35·6 mg. urea per 100 ml., that of the active kidneys being 51 mg. urea per 100 ml. Urea has, therefore, been concentrated from an external fluid by the active kidneys, but not by the inactive.

To reconcile these facts with the theory of urea concentration by water reabsorption, we believe that only one explanation is possible, and we shall show that this explanation must in turn be definitely abandoned.

It may be held that after the 20-minute washing period in Locke when the concentration of the kidneys is 34 mg. urea per 100 ml. about 16 mg. of this urea are present in the urine contained within the uriniferous tubules, and that during the remaining 45 minutes' immersion in the urea-Locke this is held there, whereas with the immersion in urea-cyanide-Locke it is practically all released, the external urea reaching then a mere passive equilibrium with the urea of the renal cells. This it might be alleged would account for the difference, so that the excess urea in the active kidneys was not concentrated from an external fluid, but was held there from the beginning of the experiment, in the urine of the tubules. This explanation involves, as a necessary consequence, that if, for the 45-minute period of re-immersion, the Locke and cyanide-Locke fluids contained no urea, then the same difference as before must be manifested at the end between the active and inactive tissues, about 16 mg. per 100 ml. should be retained by the active tissue in excess of the inactive tissue. We have, therefore, conducted a series of experiments to decide this point (which might likewise have been refuted from other available facts—but not perhaps so directly or simply).

Table VI.

Period of re-immersion in Locke after preliminary washing mins.	Active kidneys		Inactive kidneys (cyanide)		Increase in mean conc. of active over inactive kidneys mg. urea per 100 ml.
	Av. wt. of single kidney mg.	mg. urea per 100 ml. after re-immersion	Av. wt. of single kidney mg.	mg. urea per 100 ml. after re-immersion	
20	26	21	27	18	+3
20	22	19	23	18	+1
20	30	12	33	13	−1
45	25	9	30	8	+1
45	19	13	20	10	+3
45	20	13	23	12	+2

In the above experiments six pairs of kidneys were taken and washed in Locke for 20 minutes, six kidneys, one from each pair, being then removed to cyanide-Locke. These and the remaining six in ordinary Locke were washed for a further period of 20 or 45 minutes. The fluid was renewed a few times in this latter washing.

The mean difference at the end between the active and inactive kidneys is 1 mg. urea per 100 ml.

Six pairs of kidneys were selected for each experimental set and washed for 20 minutes in Locke, then three re-immersed in Locke and the companion tissues in cyanide-Locke for another 45 minutes, the conditions being therefore as before, except that the Locke for re-immersion contained no urea, and during the second period of 45 minutes was changed twice to reduce the external urea concentration to an insignificant figure. The results of these experiments are contained in Table VI. The average concentration of the active kidneys at the end of the period is 11·7 mg. per 100 ml. and that of the inactive is 10·0 mg. per 100 ml. The average difference is therefore only 1·7 mg. urea per 100 ml., whereas 16 mg. would have to be accounted for. Table II also shows the results of a similar set of experiments with a 20-minute period of re-immersion instead of 45 minutes. The mean urea value for the active kidneys was 17 and for the inactive 16 mg. per 100 ml. Over the whole period of 45 minutes' re-immersion, therefore, there is—for the purpose of the present discussion—no significant difference between active and inactive kidneys.

It is certain, therefore, that the large difference between the active and inactive tissues after re-immersion in urea-Locke is not due to the mere liberation of urea held up in the tubule lumen by the action of cyanide.

A point that may be mentioned in connection with the immersions of the kidneys in Locke and cyanide-Locke is that the weights of the kidneys alter. We have already studied this in some detail, and with results of much interest to be described in a subsequent communication. Here it may be said that if the original washing in Locke has been for 30 minutes with a subsequent 30 minutes' immersion in Locke or cyanide-Locke (cyanide concentration—0·02 % KCN) the difference between the final weights of the kidneys active and inactive, is less than 5 %. (This applies to the experiments of Table IV.) When the original washing in Locke was only for 20 minutes, with subsequent 45-minute immersions in Locke or cyanide-Locke, as in the experiments of Table V, the mean difference between the active and inactive kidneys was found to be about 7 %. The active kidneys summarised in Table V lost 10 % of their weight during the 45-minute re-immersion and the inactive lost 3 %. These changes in volume however will not influence the nature of the results obtained, since it is concentration equilibria we are dealing with and these are nearly reached at 45 minutes. If however the volume changes are reckoned as exercising the maximum conceivable effect, in a manner of argument opposing the above, then

if we calculate the final concentrations of the kidneys in terms of the weights before instead of after immersion in the urea-Locke or urea-cyanide-Locke, the average concentration for the active kidneys of Table V becomes 46 mg. per 100 ml. and for the inactive 35 mg. (the external fluid having a urea concentration of 40 mg. per 100 ml.).

This, therefore, could not affect the argument, the inactive kidney having still only 76 % of the urea content of the active, the latter being 15 % more concentrated in urea than the external fluid—and this after attaining to less than 80 % of the final equilibrium value.

Concerning the technical side of the analyses the opinion may be entertained that since differences between urease and xanthydrol determinations on kidney suspensions have been recorded in the literature, urea analyses of the frog's kidney are uncertain. The reason for such differences has been demonstrated above in the section on methods. The following facts may also be noted.

(1) From the Locke fluid in which fresh kidneys are immersed 95 % of the total urea originally present—as determined from companion tissues—can be recovered after 3 hours, and this amount corresponds to 97 % of the theoretically diffusing quantity. The remaining 3 % may even be accounted for by the swelling of the tissues after this period.

(2) The differences in urea concentration of the active and inactive kidneys, on which the proof turns, can be shown either from unboiled suspensions, boiled suspensions, or by xanthydrol analyses. There is no difference in the figures obtained by us using the xanthydrol method with unboiled suspension, or the urease method with boiled suspension. A slight difference existed between the absolute urea concentration as determined from boiled and unboiled suspensions with our method of analysis by urease. This difference was due to the inclusion of some "extra" ammonia formed by the urease extract on the unboiled suspension, already considered in detail in the first section.

The amount of this "extra" ammonia could be considerably increased by prolonging the time of incubation of the suspension with the urease extract and also by increasing the concentration of the extract itself.

From the existence of this "extra" ammonia Rehberg has even invented the conception that one moiety of urea in the frog's kidney exists in a freely diffusible form—such as was given by the xanthydrol measurements—and another as "bound" urea which could be slowly acted upon by the ferment urease and in quantity corresponding to this "extra" ammonia.

The freely diffusible form of urea was present—according to Rehberg—in quantities little if at all greater than that of the fluid with which he perfused the kidney. The truth is however that the urea in the blood of the normal frog is only 60–70 % of the true and freely diffusible urea of the kidney, and this is so whether we analyse the urea by the xanthydrol method or by the urease method—employing the latter correctly—or whether in fact we determine the urea which has freely diffused into an external Locke solution.

Not only does this relationship of blood urea to renal urea normally exist, but if we isolate the kidney and wash it so that its concentration is well below the normal blood level and then re-immerse it in Locke solution containing urea of similar or lower concentration to that of the blood, the concentration relationships of blood and renal tissue are re-established, the urea-Locke solution in this case replacing the plasma.

The concentration of urea from an external urea-Locke being demonstrated, the question arises, where is the urea stored? Does a gradient exist across the tubule cells reaching the maximum concentration in the urine or does all the

stored urea pass into the urine, the concentration of the cells being that of a passive equilibrium with the external fluid? The average urea figures found for the winter frogs was 55 mg. per 100 ml. blood and 82 mg. per 100 ml. kidney. If we assume that the urea in excess of the blood is all present in the urine, and that the urine occupies—as a maximum value—5 % of the whole tissue, then the urea in the urine must be 11 times more concentrated than the blood, which is a not improbable figure for the urine concentration when this is slowly excreted. It is possible, therefore, that all the urea concentrated from the external fluid exists in the urine. This is in fact a point of view we had previously taken in relation to substances actively secreted, and that there was no evident reason for the supposition that such substances must be concentrated within the cells, though this possibility must be allowed pending further proof.

In conclusion it may be noted that whereas in the present communication we have shown that for a secreted substance the active isolated frog's kidney comes into equilibrium with a much higher concentration than the inactive (and higher than the external fluid), the reverse effect was demonstrated for an absorbed substance. The absorbed substance in question is sodium sulphate, which in about nine cases out of ten appears in the urine of the frog (*Rana temporaria*) with only one-third or less the plasma concentration.

SUMMARY.

1. The concentration of urea from an external urea-Locke solution by the active isolated kidney of the frog has been definitely proved.

2. The proof consists in washing pairs of fresh kidneys in Locke for 20 minutes, retaining one member of each pair for a grouped analysis, the companion tissues being immersed for 45 minutes in urea-Locke, containing 40 mg. urea per 100 ml., and subsequently analysed. After the initial washing the mean urea concentration was 34 mg. per 100 ml. and after re-immersion in urea-Locke it rose to 51 mg. urea per 100 ml. When the re-immersion was made in urea-cyanide-Locke—containing also 40 mg. urea per 100 ml.—the concentration found was 35·6 mg. urea per 100 ml.

A large number of similar experiments were also carried out in which the external concentrations *etc.* were varied. The results in all cases supported the above findings.

3. When the re-immersions after the initial washing were made for 45 minutes in Locke or cyanide-Locke containing no urea, the mean difference of urea content between the active and inactive tissues did not exceed 2 mg. urea per 100 ml.

4. Changes in the volume of active and inactive (cyanide) kidneys under the conditions examined have no appreciable effect on a consideration of the results.

5. Xanthydrol determinations of the urea in the frog's kidney gave identical results with the urease method used. This latter was subjected to a detailed examination in so far as it could have any bearing on the results obtained.

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