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ANALYSIS OF THE DISTRIBUTION OF SODIUM, POTASSIUM AND OSMOTIC PRESSURE IN THE URINE OF CRAYFISHES

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INTRODUCTION

Studies of ion concentrations and osmotic pressures in the crayfish antennal gland have revealed a major discrepancy. The osmotic pressure of the urine in the tubular portion of the gland falls (Riegel, 1963). However, the sodium and potassium concentrations measured there exceed the osmotic pressure (Riegel, 1965). To complicate the situation further, studies of inulin excretion indicate that water absorption occurs in the distal tubule. Further, amino acids become concentrated in the distal tubule (Riegel, 1966*b*).

There are two apparent explanations for this discrepancy: one, the original osmotic pressure measurements were in error. Two, a major portion of the particles in the distal tubule are present in an osmotically inactive form. During investigations of the latter possibility it was discovered that formed bodies are found in all parts of the antennal gland (Riegel, 1966*a*, *b*). It is possible that the formed bodies represent a site of localization of osmotically active particles. Therefore studies were made of the possible association of sodium and potassium with the urinary formed bodies. The results of these and other studies related to the problem are presented here.

MATERIALS AND METHODS

Specimens of *Austropotamobius pallipes pallipes* (Lereboullet) were used. Most of the crayfishes were collected in a local stream (River Misbourne near Denham, south Buckinghamshire). Others were obtained from a commercial supplier. No differences were seen between the two groups of crayfishes. The animals were kept in running tap water and they were not fed.

Micropuncture samples were centrifuged in order to separate the formed body and fluid portions of the urine. For this purpose microcentrifuge tube holders of the type illustrated in Fig. 1 A were constructed. They consisted of a hollow PVC flask stopper through the bottom of which a small hole was drilled. A short length of 3 mm. (inside diameter) Pyrex glass tubing was drawn in a flame and thrust through the hole. This melted the PVC, sealing the tubing in place on cooling. Melted sealing wax was then allowed to drip into the hollow portion of the flask stopper. This served both to increase the rigidity of the microcentrifuge tube holder and to equalise weights between different holders.

Microcentrifuge tubes were capillaries constructed from Pyrex glass and drawn to a fine tip at one end. They were sealed into the holders with sealing wax. The whole

was then used as a braking pipette. Urine samples were taken up between columns of liquid paraffin. The fine tip of the capillary was sealed in a microflame. The holder was put into a shield of a centrifuge rotor and spun at 1000 to 5000 RCF from 10 min. to 1·5 hr.

The technique of gel filtration was used in an attempt to separate the formed bodies from ions in the fluid surrounding them. The gel columns and methods used were identical to those described by Riegel (1966*b*). The eluants used were 90 and 180 mM/l. KCl. The pH was varied between 5 and 8 with appropriate quantities of KOH.

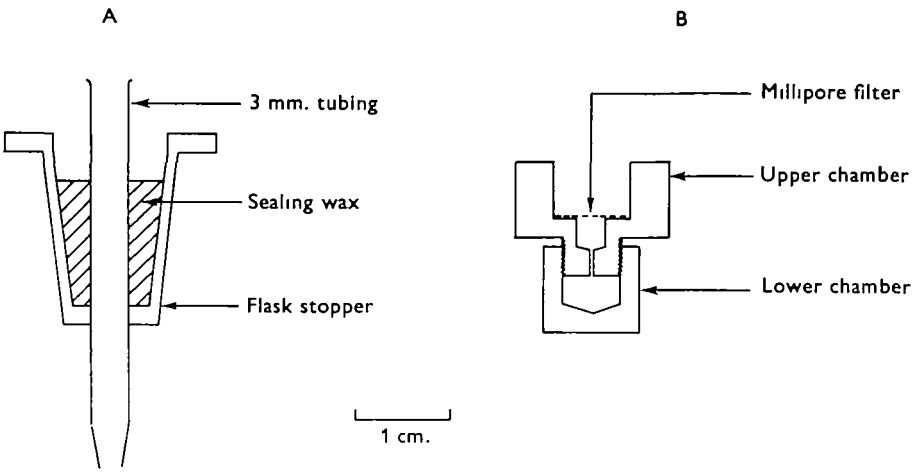


Fig. 1. A. Details of the construction of a microcentrifuge tube holder.
B. Details of construction of a filter holder for Millipore filters.

Some urine samples were filtered through PVC filters (Millipore 'Polyvic') of 0·6 μ pore diameter in order to separate the formed bodies from the fluid surrounding them. Perspex rod ($\frac{5}{8}$ in. diameter) was machined to provide a two-piece filtration apparatus like that illustrated in Fig. 1 B. A filtration device was assembled and used as follows: the bottom portion was filled with light liquid paraffin (sp.gr. 0·830–0·870). It was then screwed into the top portion, forcing the liquid paraffin into the top portion. A small (7 mm.) disk of the filter material was punched out using a size 5 cork borer. The disk was carefully inserted into the top portion to avoid trapping air bubbles. The remainder of the upper portion was filled with heavy liquid paraffin (sp.gr. 0·865–0·890). A measured quantity (*c.* 0·05–0·2 μl.) of micropuncture sample was deposited on to the surface of the filter under the paraffin. The whole device was placed in a shield in a swing-out rotor of a centrifuge. The bottom portion of the apparatus was made to just fit the internal diameter of the shield. Filtration was carried out for 1–2 min. at 1000–3000 RCF. The portion which passed through the filter was collected for melting-point analysis. During filtration the light paraffin was displaced from the bottom portion between the threads into the centrifuge shield. This process appeared to be necessary for passage of the sample through the filter.

The above technique proved useful for the crude separation of fluid and formed-body fractions of urine. This was satisfactory for relative melting-point analyses. However, attempts to utilize the technique for ion analyses proved inconclusive. This

appeared to be due to two factors: first, the amount of fluid retained in the filter was dependent upon the time taken for the sample to pass through the filter. This in turn was dependent upon the number and size of formed bodies in the sample. Secondly, some of the formed bodies passed through the filter. They appeared to be larger than the pores in the filter, but apparently they are flexible. The filter pore size was varied (0·1 μ and 0·45 μ cellulose acetate filters were also used). The smaller pore sizes resulted in slower passage of the sample through the filter. This resulted in greater retention of fluid on the filter.

Micropuncture techniques and methods of handling and analysing samples for potassium and sodium were essentially the same as those reported earlier (Riegel, 1963, 1965). Melting-point analyses were made using a modification of the comparative method of Gross (1954). This method requires relatively large samples (0·05–0·1 μl.), but several samples can be determined simultaneously.

Table 1

A. Concentrations of sodium and potassium in formed-body (*fb*) and supernatant (*s*) fractions of centrifuged micropuncture samples. B. Concentrations of sodium and potassium in labyrinth samples deposited in distilled water prior to centrifugation. The standards were 200 and 7 mM/l., respectively, for sodium and potassium concentrations.* Abbreviations: COEL = coelomosac. LAB = labyrinth, DDT = distal part of the distal tubule,. STD = standard

A

Sample	COEL		COEL		LAB		LAB		LAB			
Fraction	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*		
Na (mM/l.)	65·4	180	0	149	150	178	62·5	150	64·9	169		
K (mM/l.)	0	14·6	44·0	16·5	—	—	26·8	18 5	58·6	23·9		
Sample	DDT		DDT		DDT		DDT		DDT		DDT	
Fraction	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*
Na (mM/l.)	238	238	150	183	118	154	125	130	80	123	0	180
K (mM/l.)	—	—	—	—	24·0	24·0	4·37	23·8	19·6	25·2	62·0	28·8

B

Sample	LAB		LAB		LAB		LAB		STD	
Fraction	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*	*fb*	*s*
Na (mM/l.)	0	0	0	0	5·42	0	0	0	0	0
K (mM/l.)	1·95	0	1·78	0	3·00	0	1·95	0	—	0

* Data points indicated by 0 represent samples in which it was determined that no sodium or potassium was present. Data points represented by a dash (—) indicate that no determination was made.

RESULTS

Table 1 A summarizes the results of analyses in which urine samples from various parts of the antennal gland were centrifuged. Centrifugation produced characteristic results. In samples removed from the labyrinth and coelomosac the formed bodies moved downward. In distal tubule samples the formed bodies moved upward. This confirms observations made by Riegel (1966*b*). It was not possible to effect complete separation of the formed body and fluid fractions of the urine. The sodium and potassium concentrations of the formed-body fractions shown in Table 1 A are due in part to trapped supernatant.

The results shown in Table 1 A indicate that there is less sodium (and in most cases, more potassium) associated with the formed bodies than with the supernatant. However, they fail to show a quantitative relationship. This is possibly due to unknown quantities of fluid trapped in the formed body fraction. Gel filtration was used to attempt a more complete separation of formed bodies and supernatant. The results of these experiments are shown in Table 2. It can be seen that sodium in urine samples passes through the gel columns in a manner similar to standard sodium chloride solutions. This may indicate that most of the sodium in the urine is outside the formed bodies. There was no evidence of disruption of the formed bodies in the eluant. Urine samples were deposited in the eluant and observed under the microscope. The formed bodies did not appear to swell or burst.

Table 2

Sodium in drops of eluant collected from gel-filtration columns after passing micropuncture samples and standard NaCl solutions through the columns. Amounts of sodium expressed in mμM. Abbreviations: %R = percentage recovery of the sodium estimated to have been put into the column (see text for full explanation), PDT = proximal part of the distal tubule, others as in Table 1.

Drop number...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	%R
Sample															
STD	—	—	—	—	1·2	6·2	10·0	0·8	—	—	—	—	—	—	90
PDT	1·2	—	—	—	—	2·8	9·2	1·5	—	—	—	—	—	—	86
PDT	—	—	—	—	0·4	0 4	3·9	3·9	0·4	—	—	—	—	—	60
PDT	—	—	—	—	—	—	3·9	7·7	1·6	—	—	—	—	—	78
DDT	—	—	—	—	—	—	2·4	8·5	0 8	—	—	—	—	—	85
DDT	—	—	—	—	—	—	3·1	6·6	0·8	—	—	—	—	—	85
STD	—	—	—	—	—	—	—	—	—	—	—	14·8	12·0	—	100
PDT	—	—	—	—	—	—	—	—	—	—	—	5·6	6 8	0·4	75
PDT	—	—	—	—	—	—	—	—	—	—	1·6	10·0	5·6	0·8	100
LAB	—	—	—	—	—	—	—	—	0·8	0 0	1·6	7·2	3·6	—	74

As shown in the column marked '%R', the recovery of sodium in the standards was better than the recovery of sodium in the urine. It is possible that some sodium remains behind in the larger formed bodies which were trapped on the surface of the gel. The '%R' values was devised as follows: equal aliquots of a micropuncture sample were passed through the gel column and were analysed directly. The percentage recovery was then determined as the total sodium recovered from the column divided by the total sodium in the unfiltered sample.

In some instances formed bodies passed through the gel columns and were recovered in the first or second drop. In only one case (see Table 2) was there a significant quantity of sodium in the first or second drop. In all of the experiments listed in the table the pH was maintained at 7. In a large number of experiments, not shown, the pH was varied between 5 and 8. Rather surprisingly, this had no noticeable effect on the results. The experiments not shown were those in which measurements of unfiltered sodium were not made. However, the total amounts of sodium were comparable to those shown in Table 2.

Attempts were made to analyse for potassium in a manner similar to that for sodium. However, a satisfactory eluting medium could not be found. There was an unknown source of interference (with flame emission spectrophotometry) which resulted in potassium recovery values 2–3 times the amount of potassium put into the gel

columns. It was decided to attempt another means of determining whether potassium is associated with the formed bodies. Samples of labyrinth urine (the source of the greatest number of formed bodies in the crayfishes used) were deposited in 1 ml. of distilled water in a 5 ml. centrifuge tube. After an estimated 1–2 μl. had been collected, the sample was centrifuged. Equal quantities of formed body and supernatant fractions were then diluted to 5 ml. and anlyased for sodium and potassium. As shown in Table 1 B a small amount of potassium was present in the formed-body fractions in all samples. No potassium was found in the supernatant. Further, no sodium was found in the supernatant. In only one case was sodium found in the formed body fraction.

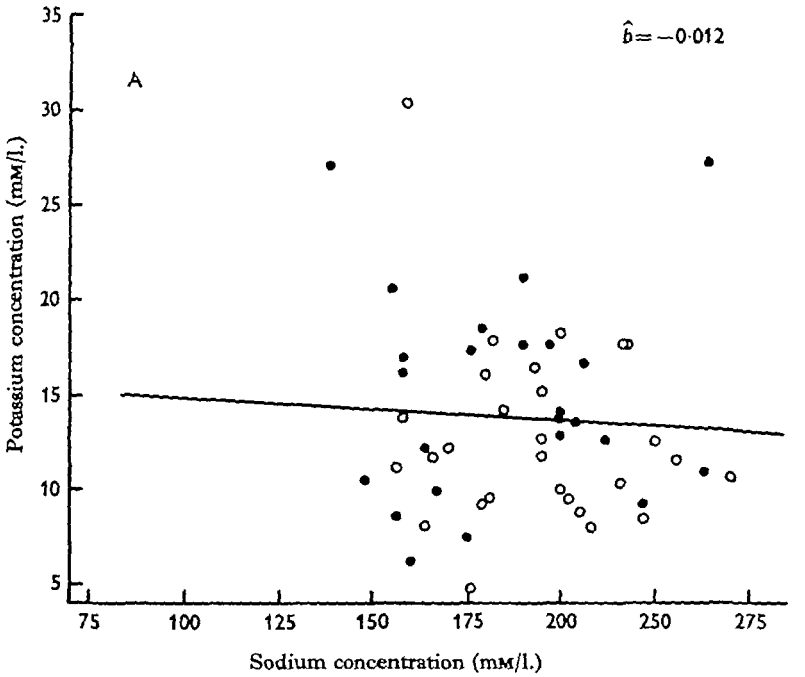
Table 3

A. Resumé of determinations of melting-points and sodium and potassium concentrations of urine removed from a series of twelve crayfishes. Concentrations are expressed in mM/l. B. Comparison of urine:blood (U/B) ratios for osmotic pressure (OP), potassium (K) and sodium (Na) in urine of the crayfishes listed in A. For comparison, data from previous studies (Riegel, 1963, 1965) are also shown. C. Osmotic pressures of micropuncture samples from three crayfishes which were either unfiltered (UF) or filtered (F) through 0·6 μ pore diameter Millipore 'Polyvic' filters.

						Distal tubule		
		Blood	Coelomosac	Labyrinth	Proximal tubule	Proximal	Distal	Bladder
				A				
OP	Mean	225	210	223	214	210	214	30 80
	Range	211–240	161–258	189–256	146–280	148–242	158–256	13·0–60·3
	S.D.	±10	±33	±20	±34	±26	±25	±14·50
	No.	10	11	11	11	10	10	11
Na	Mean	208	192	189	188	176	151	11·20
	Range	172–240	156–231	148–238	138–224	125–252	90·4–197	2 50–62·5
	S.D.	±22	±26	±30	±28	±38	±30	±6·9
	No.	11	10	11	11	12	12	10
K	Mean	4·230	13·70	14·70	16·40	21·60	25·90	0·680
	Range	1·99–7·23	8·14–30·4	8·72–27·2	6·64–27·6	9·47–45·3	13·4–43·2	0·25–1·69
	S.D.	±1·720	±6·400	±5·100	±5·900	±9·600	±9·600	±0·390
	No.	11	10	11	11	12	12	12
				B				
OP	Present	—	0·932	0·997	0·983	0·801	0·971	0·141
	1963	—	1·030	0·890	—	0·830	0·618	0·093
Na	Present	—	0·942	0·926	0·911	0·850	0·720	0·092
	1965	—	0·834	0·831	0·773	0·782	0·755	0 059
K	Present	—	4·160	4·180	4·720	5·820	6·800	0·176
	1965	—	2·610	2 410	1·900	2 720	2·480	0·283
				C				
OP	UF	231	226	219	219	200	216	—
	F	173	173	232	207	230	186	—

From the foregoing results it appears that most of the sodium in the urine is free to diffuse. It may or may not be in the formed bodies. It is therefore unlikely that the discrepancy between osmotic pressure and ion concentrations is due to localization of sodium in the formed bodies. Potassium is associated with the formed bodies, but the relative amount cannot be ascertained. It is not possible to determine from these results if the high potassium content of the urine is due to potassium in formed bodies.

The results shown in Tables 1 and 2 indicated that the low osmotic pressures



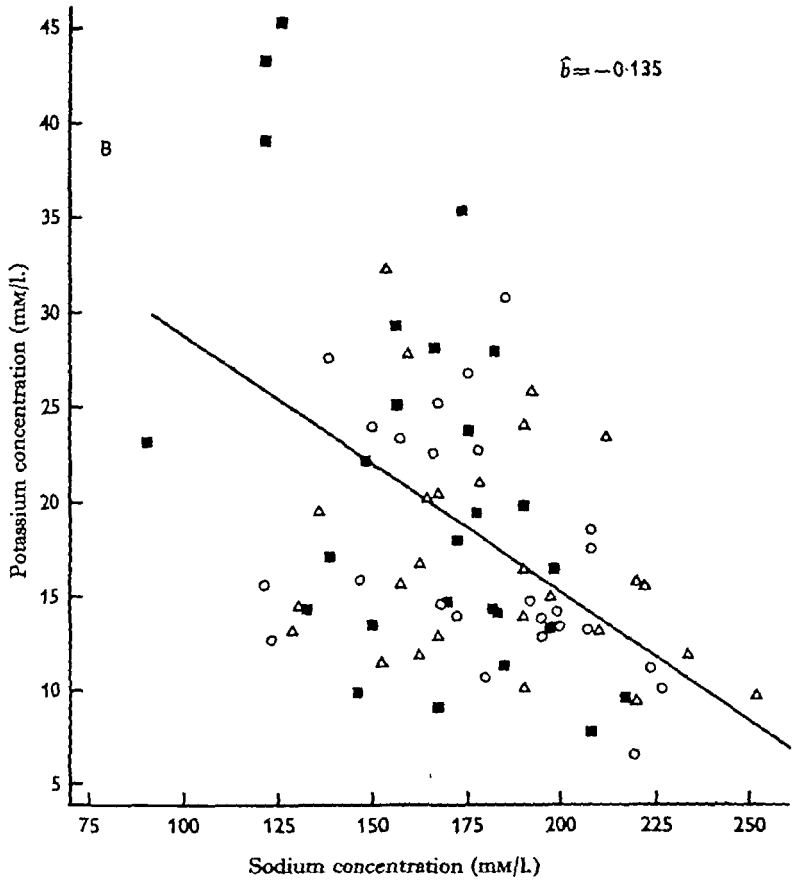


Fig. 2. For legend see facing page.

measured in the distal tubule were not due to localization of osmotically-active particles within formed bodies. Therefore, it was decided to measure the osmotic pressure of urine taken from a new series of crayfishes. In this series the samples were thoroughly mixed by picking them up and blowing them out of the sampling pipette several times. Simultaneous measurements of sodium and potassium concentration were also made.

As shown in Table 3A the osmotic pressure of the urine remains much the same throughout the antennal gland. There is no fall in the distal tubule as shown previously (Riegel, 1963). The potassium concentration rises in the distal tubule and, concomitantly, the sodium concentration falls. Such a distinct fall in urine sodium concentration in the tubule has not been observed previously. However, comparable urine : blood (U/B) ratios for sodium (Table 3B) have been seen previously (see Discussion). The possible reasons underlying the discrepancies in tubular osmotic pressures and sodium concentrations will be discussed later.

The data shown in Table 3A seemed to suggest an inverse relationship between sodium and potassium concentrations in the tubule. Therefore, potassium and sodium concentrations were plotted as shown in Fig. 2. Each plot represents a paired sodium and potassium determination. The slope of the regression shown in Fig. 2A does not differ significantly from zero. The slope of the regression shown in Fig. 2B does differ significantly from zero ($t = 3{\cdot}86$, $P = <0{\cdot}01$). Therefore, there appears to be an inverse relationship between potassium and sodium in the tubular parts of the crayfish antennal gland.

DISCUSSION

In the studies reported here it has been possible to observe crayfishes collected locally throughout most of an annual moult cycle. These observations have revealed differences which have been noted previously, but which have never been seen to fit a pattern.

The labyrinths of the antennal glands of crayfishes collected during the winter and spring were often a vivid green. These crayfishes were usually diuretic; the whole antennal gland was swollen with fluid. Micropuncture samples from the labyrinth rarely contained the characteristic large formed bodies. Large formed bodies were only infrequently observed in micropuncture samples other than those from the distal tubule. From the condition of the exoskeleton and the size of the gastroliths these crayfishes were judged to be in late intermoult (C) and premoult (D) stages of the moult cycle.

After moulting in the late spring and early summer (May–July) the labyrinth turned to a yellow colour. Large vesicles were once again observed in labyrinth micropuncture samples. The antennal gland appeared to contain less fluid (i.e. micropuncture samples were more difficult to obtain). The significance of these changes in colour of the layrinth and of the diuresis is unknown. It hardly seems likely that they are only accidental concomitants of moult-cycle changes.

The sodium and potassium concentrations summarized in Table 3A are somewhat

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Fig. 2. A. Potassium concentrations plotted against sodium concentrations of micropuncture samples from the coelomosac (open circles) and labyrinth (closed circles). B. Potassium concentrations plotted against sodium concentrations of micropuncture samples from the proximal tubule (open circles), proximal part of the distal tubule (open triangles) and distal part of the distal tubule (closed squares). Data derived from the present study and from Riegel (1965).

at variance with earlier determinations (Riegel, 1965). Except in the distal part of the distal tubule the average sodium U/B ratios are higher in the present group of crayfishes (see Table 3B). Furthermore, the potassium U/B ratios are also elevated in the present group of crayfishes. The concentrations of sodium in the distal part of the distal tubule are lower in the present group of crayfishes than in those reported on earlier (Riegel, 1965). However, if the sodium U/B ratios are compared the difference is not statistically significant.

The crayfishes studied in 1965 were intermoult summer animals. The crayfishes studied here were late intermoult and premoult (winter–spring) animals. Thus the differences in potassium and sodium concentrations in blood and urine and the U/B ratios, may be related to the moult cycle. The average blood sodium concentration in summer crayfishes is higher than in winter–spring crayfishes. The same is true of blood potassium level. With the exception of the distal tubule the sodium and potassium concentrations in the urine are similar in the two groups of crayfishes. In fact, this observation is rather puzzling. It must be presumed that most of the sodium in the urine is derived from the blood. Why should the blood-related concentration be higher in one group of animals? Does this reflect the diminution of formed bodies?

The winter–spring group of crayfishes appeared to be diuretic. As shown by Riegel (1961) diuresis in crayfishes results in increased sodium excretion. However, this need not be reflected in increased sodium U/B ratios. Furthermore, the sodium U/B ratios in the distal part of the distal tubule are quantitatively similar in summer crayfishes and in winter–spring crayfishes.

Urine removed from most parts of the antennal glands of winter–spring crayfishes had few large formed bodies. In fact, large formed bodies were consistently found only in the distal part of the distal tubule. It is possible that the elevated sodium U/B ratios are a reflection of this absence of large formed bodies. Studies reported here indicate that no appreciable quantities of sodium are localized within the formed bodies. However, they do not provide a basis for saying that no sodium is associated with the formed bodies.

When the osmotic pressure U/B ratios of the present and previous (Riegel, 1963) crayfishes are compared, rather than the actual values, the differences are not so great, except with regard to the distal part of the distal tubule. The reason for this marked difference is not readily apparent. At first it was thought that there might be a moult-stage (winter–summer) difference. However, confirmatory osmotic pressure measurements have been made on summer (July) animals, all of which had yellow labyrinths and no gastroliths. The average value of the blood osmotic pressure was about 10 mM/l. NaCl less than those values shown in Table 3A. However, the urine osmotic pressure values were within the range determined for winter–spring crayfishes.

At the time of the original osmotic pressure measurements the writer was unaware of the existence of formed bodies in any part of the antennal gland other than the labyrinth. Consequently, no special precautions were taken to mix samples prior to making melting-point determinations. It was therefore thought possible that the presence or absence of formed bodies might alter the melting-point. Accordingly, samples of blood and urine were removed from crayfishes. The samples were mixed thoroughly and one half was taken up in a melting-point capillary and frozen on solid carbon dioxide. The other half of the sample was filtered through a 0·6 μ 'Polyvic'

filter. The melting-points of filtered and unfiltered samples were determined. As shown in Table 3C the results indicate that formed bodies alter the melting-points of urine samples. In some cases (blood, coelomosac, proximal tubule and distal part of the distal tubule) the filtrate has a lower osmotic pressure than the unfiltered sample. Filtered samples from the labyrinth and proximal part of the distal tubule samples had a higher osmotic pressure than unfiltered samples.

If U/B ratios for osmotic pressure are computed by using the average value for unfiltered blood and the average value for filtered urine from the distal part of the distal tubule, an average of 0·790 results. This is below that value determined for the present group of crayfishes (Table 3B), but still higher than the U/B value determined from data published in 1963. Therefore, these results shed little light on the problem. One interesting feature of the data shown in Table 3C is a consistent iso-osmoticity of filtered blood and coelomosac urine.

One further observation bears on the problem of osmotic pressure and formed bodies. It is possible that the formed bodies do not contribute to the osmotic pressure as measured by the melting-point method. In previous studies (Riegel, 1966*b*, and unpublished) formed bodies were viewed under the high powers of the compound microscope. The micropuncture samples were deposited under liquid paraffin on siliconed microscope slides. During long periods of observation (8–10 hr.) bacteria would accumulate in the samples. These were killed by quick-freezing the slides on solid carbon dioxide. This treatment had no apparent effect upon the formed bodies. Thus whatever osmotic effect the formed bodies might have when disrupted, it is likely that they are not disrupted by rapid freezing. Great care is taken to freeze samples rapidly for melting-point analysis.

The effect of formed bodies on the measurement of melting-points of crayfish urine is unpredictable. It is possible that osmotic pressure is a meaningless parameter here. The osmotic pressure of the formed bodies is probably very much at variance with that of the fluid surrounding them. Reference to Table 3A shows that even in the distal part of the distal tubule the measured osmotic pressure can be accounted for almost entirely by sodium and potassium. As shown in other studies (Riegel, 1963, 1966*b*) there are large amounts of other substances (chloride, amino acids) present.

The relationships between sodium and potassium shown in Fig. 2 are similar to those seen in the glomerular nephron of vertebrates (Berliner, 1959/1960). For example, in the rat (Malnic, Klose & Giebisch, 1964) there is only a slight fall in urine potassium and sodium in the proximal tubule. In the distal tubule the potassium rises steeply, whilst the sodium falls. In *Necturus* (Bott, 1962) the potassium and sodium concentrations of the urine in the proximal tubule approximate to those of the plasma. However, in the distal tubule the sodium concentration falls sharply, whilst the average potassium concentrations approximate to those of the plasma. (There was a very wide scatter in the distal tubule potassium data as commented upon by Bott, 1962).) The present results differ from those generally obtained in vertebrates. In the crayfish the potassium concentrations in the proximal parts of the antennal gland (coelomosac and labyrinth) are consistently in excess of blood levels. However, in a brief report by Oken & Solomon (1960) it was indicated that the potassium concentrations in the proximal tubule of *Necturus* could be 1·5 times the plasma concentration.

It is difficult to assess the significance of the inverse sodium:potassium relationship

in the tubule. In mammals Berliner (1959/1960) has proposed an exchange between sodium and potassium. Further, Berliner, Kennedy & Orloff (1951) have suggested a potassium:hydrogen ion exchange. In both mammals and amphibians (Richards, 1938) acidification occurs in the distal tubule. Additionally, in mammals acidification also occurs in the proximal tubule (Gottschalk, Lassiter & Mylle, 1960) where the sodium and potassium concentrations in the urine are the same as in the plasma. As yet there have been no measurements of the site of acidification in the antennal gland. However, there is presumptive evidence which implicates regions distal to the labyrinth (Riegel, 1966*b*).

In crayfishes potassium is associated with formed bodies. Formed bodies have been isolated from the frog nephron (Riegel, 1966*a*). It would seem reasonable to expect that formed bodies in the nephron are not confined to frogs amongst the vertebrates.

SUMMARY

1. Osmotic pressures of urine removed from the crayfish antennal gland have been re-measured. The results fail to confirm those obtained earlier which indicated a marked fall in the distal portion of the antennal gland. The possible reasons for this discrepancy are discussed.

2. No significant fraction of the sodium in the urine is localized within the formed bodies found there. Potassium is localized within the formed bodies, but the relative amount cannot be ascertained.

3. There is an inverse relationship between sodium and potassium concentrations in the tubular (distal) portions of the antennal gland. No such relationship exists in the coelomosac and labyrinth (proximal) portions of the gland.

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