THE UPTAKE* OF INORGANIC ELECTROLYTES BY THE CRAYFISH†

By N. S. RUSTUM MALUF

(From the Department of Zoology, Johns Hopkins University, Baltimore, Maryland, and
the U. S. Fish Commission, Woods Hole, Massachusetts)

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INTRODUCTION

Ever since the latter part of the nineteenth century botanists have realized that plants are capable of accumulating minerals from fresh water, against concentration gradients, and have more recently made first attempts at unravelling the mechanism of the process (see for instance Hoagland, 1923; Hibbard *et al.*, 1926; Steward, 1933; Jacques and Osterhout, 1935; Rosenfels, 1935; Lundegårdh, 1937 and earlier). As far as the writer is aware, the first demonstration of such a kind in a fresh water member of the animal kingdom was made by Schumann (1928) who, in an apparently little read paper, showed that the newly molted amphipod crustacean, *Gammarus pulex*, can absorb Ca from very dilute solutions (1 to 10 mEq./l.). It is sometimes assumed that molluscs take up Ca^{++} from fresh water in the calcification of their shell. As far as is known, this has never been actually demonstrated and it may be just as likely that they accumulate Ca ingested with the food. Considerable preliminary work on the uptake of electrolytes by animals initiated by Koch and Krogh (1936), has recently been reviewed by Krogh (1939). In forms living in brackish water, with a saline concentration in the blood markedly higher than that of the environment, the phenomenon has been shown to occur in the shore crab, *Carcinus maenas*, by Nagel (1934).

The crayfish (*Cambarus clarkii* in this work) appears admirably suited for studies on the mechanisms of this uptake since it is hardy, can with-

* The word "uptake," as used here, implies the transport of a solute against its concentration gradient.

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stand several months without food (Brunow, 1911), exhibits a powerful capacity for Cl uptake as compared with other fresh water animals (Krogh, 1939), is of moderate size, readily obtained, and inexpensive.

The concentration of the various ions (Na^+ , K^+ , Ca^{++} , Cl^- , SO_4^{--}) in the external solutions was about 7.5 mEq./l. Their approximate milliequivalence per liter in the blood of the crayfish is 194 for Cl, 135 for Na, 14.5 for K, and 16 for Ca^{++} . The above figures for *Potamobius astacus* (the European crayfish) were obtained from the data of Griffiths (1891) and Huf (1935) since they balance up better than do those of Lienemann (1938) for *C. clarkii* and do not have the abnormally low K value (= 3.1 mEq./l.) of Bogucki (1934). The data, as a whole, indicate that the concentrations of the various inorganic electrolytes in the blood of *P. astacus* and *C. clarkii* are nearly identical.

The crayfish were put in pure dilute solutions of NaCl, KCl, Na_2SO_4 , or CaCl_2 . The attempt was made to ascertain whether the cation and anion of a given electrolyte are absorbed independently of each other and, if so, which ions are absorbed and which left behind. Since, at the outset, no Ca^{++} absorption could be detected the question arose as to whether Ca^{++} is absorbed during hardening of the new cuticle at the molting period. An attempt is also made to evaluate the hypothesis that the uptake of Cl might be of significance in the discharge of respiratory CO_2 . Evidence for the anatomical site(s) of the uptake is presented. This paper can be regarded only as a preliminary step in the solution of the mechanisms of the uptake of inorganic electrolytes by animals.

Material

The crayfish were shipped by railroad from New Orleans by the Southern Biological Supply Company, in damp *Sphagnum* moss in batches of about 35 and were received 2 days following. They were immediately put into a slanting wooden tank perforated at such a level as to permit some walking space free from the continuously renewed aerated tap water. Since crayfish can live for a few months without food, the animals were not fed and a stock was completely utilized within a month or two. Only vigorous individuals were used in the experiments.

RESULTS

1. *Site of Uptake of the Electrolytes.*—The site of active uptake of electrolytes, present in the body fluids of an animal, is commonly granted to the gills. This has been demonstrated for the anal gills of fresh water dipterous larvae by Koch and Krogh (1936). Koch (1934) found that the anal gills of the *living* larvae and the internal face of the branchiostegites of *living*

prawns will accumulate Ag if put into 0.05–0.1 per cent AgNO_3 for a short time and was the first to suggest that the main function of the anal gills is the transport of ions against concentration gradients.

If a living crayfish was briefly rinsed with distilled water and put into 0.025 per cent AgNO_3 in distilled water in the dark it died in a little over an hour and, if subsequently put into 10 per cent formalin in the light with branchiostegites removed, to aid reduction of the silver, within a few days the gill stems and gill filaments acquired a dark brown color with a metallic sheen when seen in strong light. Other structures did not behave thus even though the individual used had but lightly pigmented pleopods and venter so that changes in their color could be readily noticed. Paraffin sections were made of the gills; the only stain used was eosin. It was found that the dull brown impregnations occurred only in the cuticle (Fig. 1, C) and were easily distinguished from the sparsely scattered bright yellowish brown melanin pigment of the cuticle. The hypodermis, H, was never impregnated. The experiment was repeated with similar results. It appears that the Ag impregnation was a veritable accumulation and not merely a diffusion since an animal placed in the Ag solution about 3 hours after it had been killed by decerebration did not absorb Ag.

The uptake of inorganic electrolytes by a fasting animal does not occur by way of the alimentary tract. Enough phenol red was dissolved in the tap water to give an intense red. Even a very small drop of the water could be rendered an intense red by ammoniacal vapor. Two animals were fully immersed in such a solution and in 6 hours the kidneys, blood, alimentary tract, and the hepatopancreas were examined for phenol red by means of ammonia. The results were utterly negative and demonstrate that a crayfish does not ordinarily drink (for further evidence, see Maluf, 1937 *a*). By the same method, it was found that, in a hypertonic medium, such as 0.2 or 1.2 M NaCl, the crayfish does swallow some water; a probable explanation for this has been offered (Maluf, 1937 *a*).

The above results and the fact that, in the absorption experiments below, the medium in which the crayfish was immersed did not extend above the lower margins of the branchiostegites (the medium being pumped into the

FIG. 1. Transverse section through the gill filament of an 18 gm. crayfish (*Cambarus clarkii*). C, cuticle; G, "branchial kidneys" of Cuénot; S, hypodermal strands separating the efferent from the afferent blood sinuses; H, hypodermis—a syncytium.

gill chambers by the scaphognathites) make it very probable that, apart from Ca^{++} (see below), the electrolytes are taken up by the gill stems and gill filaments.

2. *Rôle of the Uptake of Electrolytes.* (a) *Replenishment for Saline Loss.*—There can be no doubt that the retention of Cl by the gills and the absorption of the same is an active process from the mere fact that a recently dead animal, even with mouth and anus plugged, loses Cl at a very marked rate while living controls from the same stock absorb the same. When an animal absorbs Cl from fresh water in which the Cl concentration is 0.197–0.216 mEq./l., while the Cl concentration of its blood is about 180 mEq./l., it appears pertinent to suppose that the process is of use to the animal. In some cases, the Cl concentration in 200 cc. of fresh water was diminished to such an extent by a 10 gm. animal within 24 hours as to yield no titrable value with the micro-methods below (*i.e.* was probably below 0.15 μM Cl) and the AgNO_3 test was only barely positive.

A crayfish in fresh water can compensate entirely for Cl lost in the urine by active Cl absorption. Thus, the daily rate of urine production by *C. clarkii* when fully immersed in fresh water is about 2.5 per cent of the wet weight (Lienemann, 1938). The Cl concentration of the urine can attain about 10 mEq./l. under normal conditions (Lienemann). The latter value is doubtless high since a correction was not made for the notable amount of water lost from the urine during the suction process of urine collection. Taking it at face value, however, a 10 gm. crayfish may lose, by way of its kidneys, approximately 0.038 mEq. Cl per 24 hours. A partially starved animal, with both excretory orifices sealed, can show a rate of Cl absorption from fresh water (containing about 0.2 mEq. Cl/l.) of at least 0.069 mEq./10 gm./20 hours. In higher, but nevertheless blood-inferior, Cl concentrations the rate of Cl uptake is greater (Table I).

Since Cl is not stored by tissues as far as is known, one would assume that a crayfish, in order to exhibit Cl absorption which is many times above that which could conceivably result from a mere storage of urine in the bladders, must have undergone Cl depletion. The crayfish, however, even on immediate receipt at all times of the year from the Southern Biological Supply Company, invariably displayed the capacity for Cl absorption. The reason for this may have been due to deficient dieting before shipment. The Supply Company states that “these crayfish were brought in by our collectors and placed in tanks without food about two days prior to shipment to you. . . . They had been placed in approximately two to three inches of city tap water in a large tank, one part of which was dry in order to enable the crayfish to come out and aerate themselves.” Nothing

TABLE I
Uptake of Inorganic Electrolytes by the Crayfish

Crayfish (identical nos. refer to identical individuals)	Weight in gm. and sex	Duration of experiment <i>hrs.</i>	Type of solution	Initial concn. in mEq./l.	mEq. absorbed per 10 gm. per 20 hrs.					Date
					Cl	Na	K	Ca	SO ₄	
1	14.4♂	14.2	NaCl	7.35	0.234	0.202	—	—	—	Dec. 10-11
2	18.8♀				0.075	0.172	—	—	—	
1a	14.3♀	19.0	NaCl	7.25	0.066	0.0735	—	—	—	Dec. 31-
2a	11.7♀				0.117	0.090	—	—	—	Jan. 1
1	(As above)	14.5	KCl	7.60	0.00	—	0.00	—	—	Jan. 8-9
2	" "				0.02	—	0.00	—	—	
1	" "	17.0	NaCl	7.80	0.164	—	—	—	—	Jan. 11-12
2	" "				0.100	—	—	—	—	
1	" "	16.3	KCl	8.30	0.020	—	—	—	—	Jan. 12-13
2	" "				0.00	—	—	—	—	
1a	" "	18.5	KCl	8.10	0.106	—	—	—	—	Jan. 13-14
2a	" "				0.018	—	—	—	—	
1a	" "	17.0	NaCl	7.90	0.105	—	—	—	—	Jan. 14-15
2a	" "				0.166	—	—	—	—	
1a	" "	18.0	KCl	7.80	0.031	—	0.00	—	—	Jan. 16-17
14	27.1♂	20.0	KCl	7.80	0.059	—	0.00	—	—	Jan. 18-19
15	19.4♀				0.072	—	—0.010 (a loss)	—	—	
16	29.5♂				0.027	—	—0.017 (a loss)	—	—	
14	(As above)	24	NaCl	8.40	0.0675	0.077	—	—	—	Jan. 22-23
15	" "				0.111	0.159	—	—	—	
16	" "				0.113	0.158	—	—	—	
14	" "	20.7	Na ₂ SO ₄	7.35	—	0.0356	—	—	0.000	Feb. 14-15
15	" "				—	0.0772	—	—	0.000	
16	" "				—	0.0687	—	—	0.000	
14	" "	23.5	Na ₂ SO ₄	7.40	—	0.0502	—	—	0.000	Mar. 2-3
15	" "				—	0.0345	—	—	0.000	
2 (hard-shell)	" "	23.0	CaCl ₂	7.40	0.037	—	—	0.000	—	Mar. 15-16
x (molted Mar. 7)	8.3♂				0.105	—	—	0.42	—	
30 (hard-shell)	11.1♂	22.0	CaCl ₂	7.40	0.000	—	—	—0.016 (a loss)	—	Mar. 18-19
x	(As above)				0.000	—	—	0.284	—	
40 (preparing to molt; gastro-liths fully developed)	13.5♂				0.000	—	—	0.188	—	
32 (hard-shell)	14.5	24.0	CaCl ₂	7.20	0.0575	—	—	0.000	—	Mar. 22-23
m (molted late in Dec., kept in tap water)	9.3				0.0537	—	—	0.000	—	
42 (hard-shell)	9.4				0.0354	—	—	0.000	—	
1c	9.0	12.7	NaCl	7.68	0.256	—	—	—	—	Aug. 26-27
2c	9.25		NaCl	7.68	0.243	—	—	—	—	
3c	8.0		NaCl	7.68	0.270	—	—	—	—	
4c	8.2		CaCl ₂	14.9	0.173	—	—	—	—	
5c	7.0		CaCl ₂	14.9	0.025	—	—	—	—	
1d	8.0	13.2	NaCl	8.02	0.208	—	—	—	—	Aug. 27-28
2d	9.0		NaCl	8.02	0.171	—	—	—	—	
3d	10.4		NaCl	8.02	0.160	—	—	—	—	
4d	7.0		CaCl ₂	8.45	0.00	—	—	—	—	
5d	8.6		CaCl ₂	8.45	0.104	—	—	—	—	
6d	7.3		CaCl ₂	8.45	0.122	—	—	—	—	
1d	(As above)	12.0	CaCl ₂	8.45	0.00	—	—	—	—	Aug. 29-30
2d	" "		CaCl ₂	8.45	0.144	—	—	—	—	
3d	" "		CaCl ₂	8.45	0.104	—	—	—	—	
4d	" "		NaCl	8.02	0.087	—	—	—	—	
5d	" "		NaCl	8.02	0.115	—	—	—	—	
6d	" "		NaCl	8.02	0.206	—	—	—	—	

was known, apparently, of the state of nutrition of the animals upon capture.

(b) *Release of Respiratory CO₂*.—In 1937 Ferguson, Lewis, and Smith noted that the activity of carbonic anhydrase in various marine invertebrates is greatest in the gills, less in the muscles, and practically nil in the blood. They stated that this enzyme may be of importance in the discharge of CO₂ by the gills but nevertheless pointed out that, if such is the case, it would be “hard to see how bicarbonate ions could enter the cells of the gill to be changed to CO₂, except by exchange with some anion of the gill cell, *e.g.* chloride ion. . . . To make such a scheme acceptable a mechanism for replenishing the chlorides in the gill cells would have to be found.” Lundegårdh (1933, 1937) has noted that the ratio of the rate of CO₂ evolution to anion absorption is approximately constant in the rootlets of healthy barley plants; that this ratio differs with different anions (Cl⁻, NO₃⁻, SO₄⁻); and that the absorption is continuous. On the other hand, in frogs and fresh water fishes (*i.e.* in erythrocyte-possessing animals), Krogh (1937 *a*, *b*) has found that Cl absorption is not continuous but only follows Cl depletion of the tissues. In such animals, then, Cl absorption cannot be of respiratory significance.

A comparison of the rates of CO₂ output and of Cl uptake by the crayfish (*Cambarus clarkii*) courts the apparently inevitable conclusion that Cl absorption is of no respiratory significance in this animal since the rate of CO₂ output (= *ca.* 3.0 mEq. CO₂/10 gm./20 hrs. at 25°C., according to Maluf, 1937 *b*) is many times in excess of the net rate of Cl absorption from fresh water even by the partially starved animal (= *ca.* 0.069 mEq. Cl/10 gm./20 hrs. at 25°C.). Furthermore, the Cl-absorbing capacity of the crayfish (*Potamobius astacus*) has been noted by Krogh (1939) to be “very powerful” compared with that of other fresh water animals; it therefore seems most unlikely that Cl uptake is of any respiratory significance in animals.

It was nevertheless considered of interest to measure the carbonic anhydrase activity of the gills of the crayfish and to compare this activity with that of other tissues of the same animal. The measurements were made at Woods Hole with the same apparatus used by Ferguson *et al.* The activity, *E*, is expressed per gram of fresh tissue at 15°C. at 50 per cent completion of the reaction. The tissues were pooled from six healthy animals.

The carbonic anhydrase activity of the gills, in the crayfish too, is markedly greater than that of other tissues. The blood of this invertebrate, however, shows a definite carbonic anhydrase activity.

Crayfish
(Carbonic Anhydrase Activity)

	Gills	Whole blood	Hepatopancreas	Integument (hard-cuticle)	Abdominal somatic muscles
<i>E</i>	80	26	17	15	15

Since carbonic anhydrase will accelerate any reaction in which the formation or decomposition of carbonic acid is the limiting reaction (Meldrum and Roughton, 1933) and since it may thus be of importance in the deposition and dissolution of CaCO_3 in the cuticle¹ of Crustacea, measurements were made of the carbonic anhydrase activity of the integument of the lobster (*Homarus americanus*) at pre-molt, post-molt, and hard-cuticle periods and upon other tissues of the lobster for comparison. As the following table shows, the hypodermis of the hard integument has practically the same carbonic anhydrase activity as the new integument shortly prior to molting and as the soft parts of the hardening integument following molting by about 1 week. All the activities are relatively low. The integumental samples were obtained from the branchiostegites where they are practically free from extraneous tissue.

Lobster
(Carbonic Anhydrase Activity)

	Gills	Abdominal somatic muscles	New integument prior to molt	Soft part of hardening integument 1 week after molt	Hypodermis of hard integument	Crop tissue
<i>E</i>	19-22	20	19	17	16	17

3. *Manner of Uptake of the Electrolytes from Blood-Inferior Solutions.*—It will be noted from Table I (Dec. 10-11, Dec. 31-Jan. 1, Jan. 22-23) that, in pure NaCl, Na^+ and Cl^- are not absorbed at strictly identical rates. The correspondence is quite close but the differences exceed the limits of error. In pure KCl, K^+ is not taken up at all while the rate of Cl^- uptake, by the same individuals, is generally lower than that in NaCl of approximately the same normality (Jan. 8-9, Jan. 11-12, Jan. 12-13, Jan. 13-14, Jan. 14-15, Jan. 16-17, Jan. 18-19, Jan. 22-23). This indicates that NaCl is absorbed, partly at least, in molecular form. The results also show that Cl^- can be taken up independently of the cation. In the intervals between such experiments involving back-and-forth transfer between two different

¹ The cuticle is the entire non-living component of the integument.

solutions, the animals were returned to individual battery jars containing distilled water.

In pure Na_2SO_4 , the SO_4^- is left behind entirely (Feb. 14–15, Mar. 2–3) while the Na^+ is absorbed at a rate lower than that from the same normality of NaCl (compare Jan. 22–23 with Feb. 14–15) even though the same animals were subjected to distilled water in separate jars for over 3 weeks between the NaCl and Na_2SO_4 runs. These experiments show that the Na^+ can be absorbed independently of the anion and indicate that NaCl is, to a large extent, taken up as a whole molecule; which, latter, is a confirmation of an identical statement in the previous paragraph.

From pure CaCl_2 , a hard-cuticled crayfish not preparing to molt will leave the Ca^{++} behind and generally absorb some Cl^- (Mar. 15–16, Mar. 18–19, Mar. 22–23, Aug. 26–30). An animal which has recently molted or which is preparing to molt will absorb Ca^{++} but not necessarily the Cl^- complement (Mar. 15–16, Mar. 18–19). This demonstrates that Ca^{++} can be taken up independently of the anion and that, unlike conditions with NaCl , the Ca^{++} and Cl^- are not absorbed together. The rate of Cl^- absorption, by the same individuals, from CaCl_2 is notably less than from NaCl of about the same normality (Aug. 26–30, Mar. 15–23), which corroborates the above statements that Na^+ and Cl^- are absorbed largely as NaCl . The periodic uptake of Ca^{++} during specified conditions, the unusually high rate of its uptake as compared with Na^+ or Cl^- when taken alone, and the evidence that, unlike Na^+ , it is not absorbed together with the Cl^- component, all strongly suggest that Ca^{++} is probably not absorbed through the gills but deposited directly in the cuticle, as CaCO_3 , presumably through the agency of some enzyme.

4. *Active Uptake of Calcium and the Hardening of the Integument after Molting.*—It was shown in the previous section that a newly molted crayfish will take up Ca^{++} at a rate higher than that of the other ions absorbed (Na^+ and Cl^-) and that a hard-cuticled animal will not absorb any appreciable quantity of Ca^{++} unless it is preparing to molt. It appears that a large fraction of the Ca required for the new cuticle is furnished in this way. The Ca^{++} concentration of fresh water bodies may be of importance in determining the existence of crayfish in such. After the above results were obtained, Dr. John H. Lochhead kindly called my attention to Schumann's (1928) paper in which it is shown that newly molted *Gammarus pulex*, a fresh water amphipod, absorbs Ca from concentrations as low as 1 to 10 mEq./l. and that such an animal cannot develop and breed in bodies of water with a Ca concentration lower than 0.26 mEq./l. The "hard"-

cuticled *G. pulex* does not absorb Ca. These results are in accord with those here presented for the crayfish.

One of the few crayfish which molted in the laboratory was placed in distilled water a few hours after molting. Following the molt it was lying on its side apparently incapable of normal posture; the pincers were soft and immobile; the cuticle was so soft that when the animal was lifted up its limbs fell limply; the limb segments were readily pliable; it was capable of flipping its abdomen vigorously when touched; the scaphognathites beat rhythmically; and the chelipeds, walking legs, antennae, and eyes moved frequently and 'spontaneously'. Its weight, added to that of the exuvium, showed that it had absorbed water to the extent of about 51.5 per cent of its body weight as measured 3 weeks before the molt. The animal had not been fed and showed no appreciable increase in its dimensions. In 24 hours the claws were capable of movement and hardening of the cuticle had progressed slightly. The animal attempted to assume the upright posture every now and then. In about 55 hours the upright position was held indefinitely. The cuticle appeared to undergo no further hardening even after the lapse of 2 months at which time the animal died without apparent cause. The per cent raw ash in the cuticle of the carapace of this animal was only 25.6 of the dry weight as compared with 40 or more for a hard-cuticled animal.

Another individual was kept in tap water after molting and showed a definitely more progressive picture, as regards hardening of the cuticle within a given time interval, than the above animal which was not permitted any foreign source of Ca after molting. Distilled water does not appear to have any directly deleterious effect on crayfish since they are capable of living in it indefinitely; it therefore seems most probable that the results here described are due merely to the absence of Ca from the distilled water. These data are necessarily meager but suggestive.

It is appropriate to know how much of the raw ash and organic material in the old cuticle is resorbed during molting, more especially since such data apparently have not hitherto been available for any fresh water crustacean. Knowledge concerning this was made possible partly by the fact that a crayfish was weighed a few weeks before molting; hence its normal weight was known, since a fasting crayfish decreases in weight only very slowly with time. The exuvium of the animal was dried and ashed. To obtain further pertinent data, hard-cuticled crayfish were dried, weighed, and ashed. The integument of a hard-cuticled crayfish, not preparing to molt and with a heavily melanized abdominal venter, was scrupulously

freed of all adherent material including hypodermis. The limbs were incised longitudinally and their insides scraped free of tissue. From such a preparation the raw ash and organic material (by difference) fractions were obtained. Other crayfish were dried and the per cent dry weight, of the total weight, obtained. The data are summarized as follows:

Total weight of crayfish a few weeks before molting.	17.5 gm.
Per cent dry weight of total weight.	26.0
∴ Total dry weight.	4.55 gm.
Per cent dry cuticle of total dry weight.	80.4
∴ Weight of cuticle.	3.66 gm.
Per cent ash (chiefly CaO) in the cuticle.	37.8 gm.
∴ Weight of ash in cuticle.	1.38 gm.
But weight of ash in the exuvium of the 17.5 gm. animal . .	1.325 gm.
∴ Amount of raw ash resorbed.	0.055 gm., or 3.98 per cent of the original.
Per cent organic material in the dry cuticle.	62.2
∴ Amount of organic material in dry cuticle.	2.28 gm.
But weight of organic material in the exuvium of the 1.75 gm. animal.	2.228 gm.
∴ Amount of organic material resorbed.	0.052 gm., or 2.28 per cent of the initial.

The fact that a fasting crayfish does not increase appreciably in dimensions following molting, in itself indicates that shedding involves an unnecessary loss. The fact that only about 4 per cent of the raw ash of the old cuticle is resorbed and that the animal loses about 84 per cent of the total amount of ash in the body, by far the chief mineral of which is Ca, corroborates such a view.

Methods

The crayfish was wrapped in a dry towel and left thus for a few minutes to remove excess moisture. The water in the branchial chambers and various grooves of the body was then drained by tap suction and the animal, including its branchial chambers, was irrigated with the solution like that (*i.e.* from the same stock) in which it was to be immersed. It was then put into a 15 × 7.5 cm. crystallizing dish, containing 200 cc. of the solution, with the cover supported by a 0.5 cm. (diam.) glass rod across the mouth. The solution in the dish did not extend far above the lower margins of the branchio-stegites but it was deep enough to be pumped into the branchial chambers by the scaphognathites *ad lib.* Identical crystallizing dishes without crayfish were maintained as controls. The room temperature varied from 24–27°C. In the later experiments it was not considered necessary to plug the excretory orifices (see Maluf, 1939)

or drain the bladders before introducing the animals into the solutions inasmuch as it can be shown that the maximum amount of urine that can be stored in both bladders, of a 10 gm. animal for example, contains no more than *ca.* 0.0023 mEq. Cl and much less Ca^{++} and K. Unlike *C. bartoni* (Maluf, 1939), however, Cl absorption by *C. clarkii* does not seem to be notably affected by the dental plugging cement.

Chemical Analyses.—All the methods involved the use of a Linderstrøm-Lang-Keys microburette. This was of 250 c.mm. capacity, divided into cubic millimeters, and of uniform bore as shown by measurements of the length of a drop of mercury at all levels.

Standard solutions of three different concentrations of each electrolyte, closely surrounding the encountered range, were made up and the value of an unknown ascertained by interpolation. All analyses were made in duplicate or triplicate.

Chlorin.—To 1 cc. of the medium in a 9×75 mm. test-tube was added 0.5 cc. *ca.* 0.04 N AgNO_3 in 68–70 per cent HNO_3 ; the test-tubes were capped with glass bulbs and heated in boiling water for 15 minutes; 0.1 cc. ferric ammonium sulfate was used as indicator and 1 cc. of pure ethyl alcohol was added to enhance flocculation of the AgCl . Titration was with *ca.* 0.1 N NH_4SCN . For the analysis of Cl in fresh water the same volumes of reagents were used as stated in a previous paper (Maluf, 1939). The accuracy, as shown by triplicates and recovery from standard solutions, was within 1 per cent.

Sodium plus Potassium was measured by Krogh's (1938) modification of Linderstrøm-Lang's (1936) technique. Tamworth "Glasbake" centrifuge tubes (Tamworth Associates, Inc., Needham Heights, Mass.) were substituted for the "supremax" glassware. To remove the NH_4Cl and $(\text{NH}_4)_2\text{CO}_3$ formed during the procedure, the centrifuge tubes were ignited at 330–360°C. for 2 hours. The aliquots from the supernatant liquids were drawn by means of self-improvised carefully calibrated pipettes of the constriction type with fine tips. The accuracy was within 2 or 3 per cent.

Calcium.—To 1 cc. of the *ca.* 7.5 mEq./l. solution in a 15 cc. "Glasbake" centrifuge tube was added 0.5 cc. concentrated NH_4OH and the whole heated at 50–60°C. in a water-bath for 5 minutes while stirred with CO_2 -free air. 1 cc. 0.2 N NH_4 oxalate was added; the tube was stoppered and allowed to stand overnight. Wang's (1935) method of washing was adopted except that, in our relatively pure solutions, only one washing was considered necessary. Ignition was according to the method of Siwe (1935). The residue was completely dissolved, with the aid of a fine glass rod, in 1.4 cc. 0.015 N HCl and the excess acid titrated with 0.1 N NaOH to a weak red with phenolphthalein as indicator. The air current for stirring had been rendered CO_2 -free. The accuracy was within 3 per cent.

Sulfate was measured essentially according to Øllgaard's (1934) technique adapted to suit the larger quantities used in this work. To a 2 cc. sample of the *ca.* 7.5 mEq./l. solution in a 15 cc. pyrex centrifuge tube was added 0.1 cc. 99.5 per cent glacial acetic acid. After mixing, 5 cc. of freshly made 1 per cent benzidin in sulfate-free acetone were added and the whole mixed and allowed to stand for about an hour. The tubes were then centrifuged at about 2,000 R.P.M. for 5–6 minutes; the supernatant liquid was decanted as completely as possible without disturbing the residue. 10 cc. sulfate-free acetone were added to the residue without expressly breaking up the latter and the tubes centrifuged at about 2,000 R.P.M. for 5–6 minutes; the supernatant liquid was decanted and the adherent acetone allowed to evaporate. To each residue was added 0.35 cc. *ca.* 0.1 N carbonate-free NaOH . Each tube was then individually immersed in a bath at 75–80°C. and the residue and white particles on the lower wall of the tube

completely dissolved by means of a very fine glass rod. Without permitting cooling to the extent of solidification of the formed benzidin hydrate, the tube was placed in a 250 cc. beaker containing water at about 70°C. and 4 cc. of the alcoholic ammoniacal solution added. The liquid remained *clear* and colorless. Titration, while still in the warm water beaker, was with standardized 0.1 N BaCl₂ soon after adding 1 or 2 small drops of *ca.* 0.001 per cent Na rhodizonate (Eastman) made up within $\frac{3}{4}$ of an hour before the titration. The end-point was a sharp bluish red. The accuracy was within 2 per cent as shown by duplicates and recoveries from standard solutions.

The *carbonic anhydrase activity* of the gills and various tissues was measured according to the method described by Meldrum and Roughton (1933), Brinkman, Margaria, and Roughton (1933), and Ferguson, Lewis, and Smith (1937). Extraction was performed by freezing the briefly water-rinsed tissues, macerating thoroughly while frozen, and then suspending in ten times their weight of distilled water. The extracts, with a drop of toluene, were kept in a refrigerator at about 5°C. for about 20 hours before use. The manometer bath was set at 15°.

DISCUSSION

One of the first questions which arises is whether the electrolytes are taken up as molecules or as ions. It is shown, in the present work, that the crayfish can take up the anion component of a dissolved electrolyte independently of the cation component and *vice versa*. Taking a specific example, Cl⁻ is absorbed from KCl and K⁺ is left behind. One might, however, suppose that the Cl⁻ is absorbed as HCl and that the K⁺ is left behind with the hydroxyl moiety of the water molecule. In support of such a supposition is the low electrical conductivity of certain cell walls and protoplasmic membranes, which, evidently, can permit but little dissociation (see the reviews of Osterhout, 1933, 1936). The following phenomena are against such a theory: (1) The bioelectrical effects imply appreciable permeability of the bounding membranes to certain ions at least (Osterhout, 1933²); (2) HCl and other mineral acids are known to enter living cells with difficulty, especially as compared with metallic hydrates (Jacobs, 1922; Osterhout, 1936; and others).

It is here shown that K⁺ is not absorbed but that Na⁺ is absorbed. It is thus clear that the uptake of ions is not necessarily correlated with their mobilities.

The uptake of Cl⁻ from KCl, from which the cation is not absorbed, and the uptake of Na⁺ from Na₂SO₄, in which the anion is not absorbed, probably implies two separate mechanisms: one which takes up the cation and the other the anion. The fact that Cl⁻ is absorbed generally at a faster rate in NaCl than in KCl or CaCl₂, from which the metallic ions are either

² Osterhout, W. J. V., Permeability in large plant cells and in models, *Ergebn. Physiol.*, 1933, **35**, 1012, 1013.

not absorbed (Ca^{++} , ordinarily, and K^+) or else are absorbed independently of the Cl^- (Ca^{++} in newly molted and about-to-molt individuals), indicates that Na^+ and Cl^- are taken up largely together in molecular form—which may require a third type of mechanism. Since Ca^{++} is taken up only during specified periods, and then at a notably higher rate than that generally observed for Na^+ or Cl^- when such are absorbed alone, it appears that there is a fourth type of mechanism for Ca^{++} absorption. It is possible that through the agency of some periodically secreted hypodermal enzyme, Ca is deposited as CaCO_3 at the cuticle and might not actually enter the body. Reasons are given for the belief that the Na^+ and Cl^- are absorbed by the gills.

Our results support the statement of Krogh (1937c) that “the selectivity of the transport mechanism is too high” to be readily explicable on the basis of an E.M.F. as the driving force, and Osterhout (1936) has pointed out that “if the surface charge determined the entrance of electrolytes it is difficult to see how both anions and cations could enter in equal numbers, as happens in *Valonia* [a marine alga]; for a negative charge would inhibit the entrance of anions and a positive charge that of cations.” As already stated, we have presented good evidence that Na^+ and Cl^- are taken up together as NaCl or else are taken up in such a way that they mutually aid each other. This is apparently inexplicable by surface charges.

In reference to the marine alga, *Valonia*, Osterhout (1936 and earlier) has piled up considerable evidence which suggests that the accumulation of K^+ in this organism can be explained on the basis of the higher chemical potential of K^+ , OH^- in the sea water than in the cell sap, the KOH activity gradient being due to acid metabolites of the cell sap which keep the pH of the sap markedly lower than that of the sea water. Such an explanation is admittedly inapplicable to fresh water forms (Jacques and Osterhout, 1935), in which no such differences in pH exist and in which the concentration gradients are much steeper (see also Steward, 1935, and Hoagland and Broyer, 1940).

The active transport of inorganic molecules is doubtless intimately correlated with the metabolism of the cell but no general explanation for such has as yet appeared. At present, the simplest explanation for the uptake of Cl^- from KCl (in which K^+ is left behind) is the assumption that this occurs in exchange for catabolic HCO_3^- and that the uptake of Na^+ from Na_2SO_4 (in which SO_4^- is left behind) occurs in exchange for catabolic NH_4^+ . (According to Brunow, 1911, 30–40 per cent of the total non-protein nitrogen eliminated by the crayfish is NH_3-N ; according to Delaunay, 1931 and earlier, about 60 per cent of the non-protein nitrogen in crayfish

urine is $\text{NH}_3\text{--N}$.) No mere ionic exchange, however, seems capable of explaining why the uptake of Cl from NaCl (in which both Na^+ and Cl^- are taken up) is generally greater than the uptake of Cl^- from KCl (in which the cation is left behind). The absorption of Na^+ and Cl^- is so intimately correlated as to make it evident that both ions are absorbed largely as one molecule.

Krogh (1939) has remarked that the crayfish (*Potamobius astacus*) does not take up Ca^{++} ; it appears very probable, from the present work, that he was dealing only with hard-cuticled animals not preparing to molt. The work of Irvine and Woodhead (1889) indicates that the shore-crab (presumably *Carcinus maenas*) requires CaCl_2 in sea water to harden its cuticle. Robertson (1937) has shown, by macro-analysis of samples of the natural sea water at intervals of about 7 days, that the soft-cuticled post-molt *C. maenas* absorbs notable quantities of Ca^{++} from the sea water. In addition, some Cl^- absorption was recorded. Since the diffusible, *i.e.* non-protein bound, blood- Ca^{++} of these animals is considerably higher than that of the sea water (Robertson) it is clear that even a marine crustacean can take up Ca^{++} from the exterior against the concentration gradient. The gradient, however, is relatively shallow in this case.

That the gastroliths can be of no significance in the calcification of the cuticle, due to the small quantity of calcareous material of which they are composed, has long been realized (Oesterlen, 1840; van der Hoeven, 1846-55). In *Cambarus clarkii* the raw ash which constitutes both of the apparently fully developed gastroliths is only about 3.16 per cent of the total ash in the integument of a hard-cuticled animal.

SUMMARY

1. Reasons are given for believing that the uptake of Na^+ , Cl^- , and NaCl by the crayfish occurs through the gills.
2. A crayfish in fresh water, with a Cl concentration of about 0.2 mEq./l., can, by active Cl absorption, compensate entirely for Cl lost in the urine.
3. The carbonic anhydrase activity of the gills is markedly higher than that of other tissues of the crayfish, but the equivalent CO_2 output of the crayfish is far in excess of the equivalent Cl absorption per unit time and weight and thus fails to warrant the supposition that Cl absorption is of respiratory importance.
4. The carbonic anhydrase activity of the soft integument of the lobster, before and after molting, and of the hypodermis of the hard-cuticled animal is almost identical and of the same order as that of other tissues of the lobster.

5. The concentration of the electrolytes was about 7.5 mEq./l.; *i.e.*, considerably lower than in the blood of the crayfish. Cl^- can be taken up independently of the complementary cation. Na^+ can be taken up independently of the complementary anion. K^+ and SO_4^{--} are not taken up at all. In pure NaCl, the Na^+ and Cl^- are absorbed evidently largely together. Ca^{++} is absorbed only in newly molted animals and in animals preparing to molt but is not absorbed by hard-cuticled animals not preparing to molt. Ca^{++} is taken up independently of Cl^- in pure CaCl_2 .

6. Newly molted animals absorb Ca^{++} at a rate exceeding that of the absorption of other absorbable ions (Na^+ and Cl^-) in the same equivalent concentration.

7. A crayfish utilizes the Ca^{++} in fresh water in the calcification of its cuticle. Since the animal does not swallow water, the Ca^{++} must enter through the exterior. Reasons are given for believing that, unlike Na^+ and Cl^- , Ca^{++} is absorbed directly from the exterior by the integument and does not enter the body through the gills.

8. During molting, only about 4 per cent of the raw ash and 2.3 per cent of the organic material of the old cuticle is resorbed.

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