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FURTHER OBSERVATIONS ON THE PHYSIOLOGY OF SALINITY ADAPTATION IN THE CRAB-EATING FROG (*RANA CANCRIVORA*)

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INTRODUCTION

The euryhaline crab-eating frog (*Rana cancrivora*) of south-east Asia can tolerate environmental salinities ranging from fresh water to 80 % sea water (Gordon, Schmidt-Nielsen & Kelly, 1961). The osmotic concentration of the blood plasma of the adult frog increases as the concentration of the outside medium increases; the plasma is always hyperosmotic to the medium. Increased plasma osmotic concentration is due to increased concentrations of sodium, chloride, and, especially, urea. Several interesting physiological problems arise in connection with these concentration changes in the adult frog.

The uraemia that develops when frogs are placed in high salinities must be produced by changes in rates of urea production and loss which result in net urea retention. Rates of both urine production and associated cloacal urea loss decrease as salinity increases (Schmidt-Nielsen & Lee, 1962). However, there is no information on rates of urea loss through the skin of these frogs, nor is there information on possible rates of urea production.

Sodium and chloride concentrations in plasma from frogs in fresh water are higher than those in the environment, but the reverse is true in frogs in salinities higher than 30 % sea water (Gordon *et al.* 1961). The skins of various species of frogs in fresh water actively pump sodium inwards (Ussing, 1960), but this response would seem inappropriate in *R. cancrivora* at salinities above 30 % sea water. Electrical potentials and short circuit currents measured in isolated skins of *R. cancrivora* over the range of salinities tolerated by the species were consistent with either active transport of sodium inward, even in the highest salinities, or an active transport of chloride outward (Gordon *et al.* 1961). However, these observations were made before the uraemia of *R. cancrivora* was discovered, and the solutions used for bathing the insides of the skins contained no urea. It is possible that the presence of urea might change the electrical properties of the skin of these frogs.

Finally, the large changes in osmotic and solute concentrations of the blood that occur in adult frogs as the salinity of their environment changes must influence the composition of intracellular fluids. Major changes in intracellular composition associated with salinity adaptations have been described in two species of toad, *Bufo boreas* and the euryhaline *B. viridis* (Gordon, 1965). What changes occur in the tissues of *Rana cancrivora*?

The present paper describes the results of measurements carried out to elucidate these matters.

MATERIALS AND METHODS

The majority of the work was carried out in Thailand in 1963. Adult frogs were captured at night around breeding ponds or were purchased in local markets. Both natural and laboratory environmental conditions have been described by Gordon & Tucker (1965). Frogs were maintained in covered plastic containers partly filled with water of the desired salinity (100 % sea water = 1 osmole/l.), but were not fed. Part of the bottom of each container was dry, so the frogs could leave the water. Survival under these conditions was excellent. The frogs weighed between 10 and 40 g. No attempt was made to separate the sexes.

Except as noted below, analytical chemical methods and precision of results were the same as described by Gordon (1965) and by Gordon & Tucker (1965).

Total urea loss from intact frogs was determined by measuring the increase of urea in water in which each frog was immersed. The micro-diffusion method was used for urea analyses. A frog adapted to a particular salinity was placed in a plastic container that contained a urea-free, 100 ml. aliquot of water with the same salinity. The container had a perforated lid to permit air exchange, and the frog was immersed except for its head. Urea loss over a 24 hr. period was determined by analysing water samples taken at 12 and 24 hr. A few drops of toluene were added to each container to prevent bacterial decomposition of urea; control experiments showed that such decomposition did not occur.

Oxygen consumption was determined for individual animals weighing 7–18 g. The frogs were placed in sealed plastic containers that contained a known volume of air (110–130 c.c.) and 1 or 2 cm. depth of water of the same salinity to which the frog had been adapted. Gas samples of about 0·25 c.c. were taken at 20 min. intervals with a syringe that could be inserted into the container through a serum stopper. The gas samples were immediately analysed for oxygen concentration by the method of Scholander *et al.* (1955). The accuracy of each analysis was 0·5 % oxygen or better. The frogs usually were immobile while in the jars.

Measurements of electrical potentials and short circuit currents were carried out on isolated pieces of belly skin (113 mm.^{2} in area) taken from four adult frogs adapted to 60 % sea water. Procedures were the same as were used by Gordon *et al.* (1961), with the exception that a Hewlett-Packard Model 402 vacuum tube voltmeter was used for all electrical measurements. Potential and current measurements were first made on each piece of skin with both sides bathed in an unbuffered Ringer solution made up to simulate plasma composition in frogs in 60 % S.W. (200 mM-NaCl, 9·0 mM-KCl, 320 mM-urea). This Ringer was next replaced on both sides of the skin (care being taken not to stretch the skin unduly) by a Ringer solution of the same osmotic concentration, but with the urea replaced by NaCl (160 mM). The measurements were repeated. The measurements were then made a third time with the original solutions. Five to ten minutes were allowed to pass after each change of Ringer to permit voltage and current stability to return to the preparation before measurements were made.

The analyses from which intracellular concentrations were calculated were per-

formed in Los Angeles on blood and muscle samples obtained from frogs shipped from Thailand by air. Maintenance conditions for the frogs in Los Angeles were identical to those in Thailand, except that room temperature averaged 25° C. Amino acid analyses were carried out on a Spinco Model 120 B amino acid analyser.

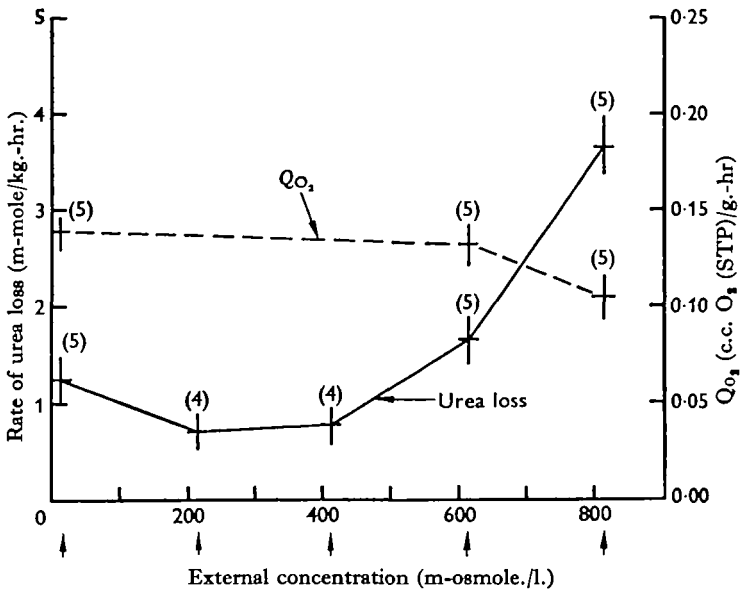

Fig. 1. Rates of urea loss and oxygen consumption of adult *R. cancrivora* fully acclimatized to different external salinities. Horizontal bars indicate means for stated number of observations; vertical bars ±2 S.E. of means. Solid line (—), rate of urea loss; dashed line (– –) rate of oxygen consumption. Arrows along abscissa mark acclimatization concentrations.

Table 1. *Rates of total urea loss and oxygen consumption of adult* Rana cancrivora *adapted to different salinities*

Salinity (% sea water)	Rates [$\bar{X} \pm$ S.E. (N)] Urea loss (mM/kg./hr.)	Oxygen consumption (c.c. O_2 (STP)/g.-hr.)
0 (fresh water)	1·2 ± 0·2 (5)	0·14 ± 0·01 (5)
20	0·7 ± 0·2 (4)	—
40	0·8 ± 0·2 (4)	—
60	1·6 ± 0·2 (5)	0·13 ± 0·01 (5)
80	3·6 ± 0·3 (5)	0·10 ± 0·01 (5)

RESULTS

There were no statistically significant variations in rate of urea loss from *R. cancrivora* over the salinity range from fresh water to 40 % S.W., but between 40 and 80 % S.W., the rate of urea loss increased markedly (Fig. 1, Table 1). The oxygen consumption results (Fig. 1, Table 1) showed a different pattern. There were no statistically significant variations in weight-specific rate of oxygen consumption over the full range of salinities studied.

The electrical properties of isolated skins of *R. cancrivora* adapted to elevated

salinities were influenced by the substitution of NaCl for urea in the fluids bathing them. In three of four skins tested the isosmotic substitution of NaCl for urea in the bathing medium produced decreases in both voltage generated by the skins and inward directed short-circuit currents flowing across the skin (Table 2). These decreases were largely restored by removal of the NaCl and replacement of the urea. The fourth skin showed an insignificant change in potential and a continuing decline in short-circuit current.

The concentrations of various substances in plasma and muscle samples from frogs adapted to different salinities are shown in Tables 3, 4 and 5.

Table 2. *Electrical properties of isolated skins of four adult* Rana cancrivora *adapted to* 60 % S.W.

	Skin potentials (mV.; inside positive)			Short-circuit currents (μA/mm.^{2} inward)		
Frog	Urea	No Urea	Urea	Urea	No Urea	Urea
1	17	8	11	0·17	0·12	0·13
2	9	3	9	0·21	0·05	0·13
3	5	1	5	0·18	0·04	0·16
4	12	13	8	0·36	0·31	0·25

Table 3. *Plasma and whole muscle concentrations in adult* Rana cancrivora *adapted to different salinities*

	Salinity (% sea water)		
	0	60	80
Substance		[$\bar{X} \pm$ S.E. (N)]	
Plasma			
Cl^- (m-equiv./l.)	113 ± 2 (5)	131 ± 2 (4)	227 ± 13 (3)
Na^+ (m-equiv./l.)	122 ± 7 (5)	181 ± 6 (5)	205 ± 11 (4)
K^+ (m-equiv./l.)	6·4 ± 0·6 (5)	6·7 ± 0·1 (5)	7·1 ± 0·5 (4)
α-NH_2N (mM/l.)	3·4 ± 0·4 (3)	—	12 (1)
Urea (mM/l.)*	40 ± 1 (5)	300 (1)	350 ± 1 (4)
Skeletal muscle (wet weight)			
Water (g./kg.)	796 ± 4 (12)	761 ± 5 (13)	749 ± 7 (10)
Inulin space (g./kg.)†	99 ± 4 (25)	100 ± 4 (5)	99 ± 5 (5)
Cl^- (m-equiv./kg.)	17 ± 1 (6)	27 ± 1 (5)	33 ± 2 (4)
Na^+ (m-equiv./kg.)	23 ± 3 (5)	35 ± 4 (5)	41 ± 4 (5)
K^+ (m-equiv./kg.)	73 ± 3 (5)	85 ± 2 (5)	86 ± 5 (4)
Non-protein nitrogen (NPN; g./kg.)	3·8 ± 0·2 (5)	9·2 ± 0·6 (5)	11·9 ± 0·3 (5)
α-NH_2N (g./kg.)	0·54 ± 0·05 (5)	1·04 ± 0·10 (5)	1·39 ± 0·10 (5)

* Data from Gordon *et al.* (1961).
† Data from Cleworth (1967).

DISCUSSION

Urea loss and oxygen consumption

When adapted to high salinities *R. cancrivora* loses urea through its skin at an appreciable rate. Schmidt-Nielsen & Lee (1962) measured rates of urea loss from the cloaca of *R. cancrivora* adapted to different salinities, and the differences between their figures and ours for total rates of urea loss represent the rates of urea loss through the skin. At salinities below about 30 % S.W., the skin is probably nearly impermeable to

Table 4. *Intracellular concentrations in skeletal muscles of adult* Rana cancrivora *adapted to different salinities*

(All amounts per kg. cell water; cf. Gordon (1965) for method of calculation.)

Substance	Salinity (% sea water) 0	60	80
Cl^- (m-equiv.)	9±2	21±1	17±4
Na^+ (m-equiv.)	16±2	26±2	32±2
K^+ (m-equiv.)	103±2	127±2	131±2
NPN (g.)*	5·3±0·1	12·7±0·1	16·8±0·1
Free α-NH_2N (g.)	0·76±0·01	1·56±0·02	2·11±0·03
Urea N (g.)†	1·1±0·1	8·4±0·1	9·8 ±0·1

* Calculated on assumption that plasma NPN = plasma urea N + plasma α-NH_2H.

† Data from Gordon *et al.* (1961). Muscle urea spaces are exactly equal to muscle water contents in all salinities (Cleworth, 1967).

Table 5. *Major ninhydrin-positive compounds in skeletal muscles of five adult* Rana cancrivora *adapted to different salinities*

(Only those compounds are listed that had a concentration of at least 2 mM/kg. in at least one animal. All concentrations in mM/kg. wet weight.)

Compound	Salinity (% sea water) 0	0	0	80	80
Taurine	8	6	17	13	20
Urea	70	12	60	100	150
Asparagine-glutamine	2·5	2·0	1·5	16	2·5
Proline	0·5	1·5	0·5	2·5	0·0
Glutamic acid	0·5	0·0	0·5	2·0	1·0
Glycine	11	5·5	5·5	17	12
Alanine	1·5	0·5	0·5	4·0	1·5
β-Alanine	0·0	0·0	5·5	9·0	12
Carnosine	1·0	5·0	2·0	11	4·5

urea, since Schmidt-Nielsen and Lee's figures for cloacal urea loss are equal to or greater than ours for total urea loss. The urea permeability of the skin of fresh-water frogs in fresh water appears to be low (Garby & Linderholm, 1954). Above 30 % s.w., the skin of *R. cancrivora* appears to become permeable to urea, and the rate of urea loss increases sharply as salinity increases (Fig. 1).

Our values for total rates of urea loss are too high for the frogs to maintain indefinitely. For example, frogs in 80 % s.w. lose 3·6 mM urea/(kg.-hr.) and would have to process 630 mg. protein/(kg.-hr.) to produce this urea (assuming protein = 16 % nitrogen). Oxidation of this amount of protein would require about 0·6 ml. O_2/(g.-hr.), or six times the rate of oxygen consumption we measured. Even the lowest mean rate of urea loss we measured would just be accounted for if the oxygen consumption of the frogs were used exclusively for protein metabolism.

It is possible that our measured rates of urea loss are unphysiologically high. The urea permeability of the skins of the experimental frogs may have been high due to the presence of toluene in the experimental containers, or perhaps due to disturbances of their mucus coverings. However, even if the measured rates of loss *were* physiological, it would still be fairly easy for the frogs to modify their behaviour so as largely to

avoid the problem. *R. cancrivora*, like most frogs, spends a great deal of its time out of water. Indeed, under some conditions it prefers being dry to being in contact with water of any normally occurring salinity (Gordon *et al.* 1961, p. 670). Suitable behavioural adjustments in lengths of time spent in and out of water could serve to maintain daily urea losses well within the limits set by the available synthetic machinery. The synthetic machinery itself is also very probably not restricted to a complete dependence on protein. Other frogs synthesize significant amounts of urea via deamination of amino acids (Janssens, 1964) and purine breakdown (Brodsky *et al.* 1965; Goldstein & Forster, 1965).

The oxygen consumption of *R. cancrivora* is within the range of that of other amphibians (Altman & Ditmer, 1964) and does not change significantly at different environmental salinities. If osmoregulation at high salinities has an extra energetic cost, either it is less than the accuracy of our measurements, or it is compensated for by reduction in other metabolic activities.

Electrical properties of skin

The changes in the electrical properties of isolated skin of *R. cancrivora* when urea is substituted for NaCl in solutions bathing the skin suggest that the presence of urea activates either an inward cation pump or an outward anion pump. In light of the many differences that may exist between isolated frog skin and that *in situ* (Ussing, 1965; Kidder, Cereijido & Curran, 1964; Brown, 1962), it is not profitable to speculate further on the significance of our electrical measurements to the process of osmoadjustment.

Intracellular osmoregulation

During adaptation to high salinities, the plasma osmotic concentration of *R. cancrivora* increases, as do plasma concentrations of sodium, potassium, chloride, amino nitrogen and urea (Table 3 and Gordon *et al.* 1961). Our measurements indicate similar changes in muscle cells.

Muscle cells in frogs adapted to high salinities show increases in intracellular concentrations of sodium, potassium, chloride and non-protein and amino nitrogen (Table 4). In addition Cleworth (1967) showed that urea spaces in the muscle of *R. cancrivora* equal total muscle water contents in all salinities between F.W. and 80 % S.W. Intracellular urea concentrations, therefore, increase with increasing salinities since they always are equal to extracellular concentrations.

These increases in intracellular concentrations could come about partly by a decrease in cell volume or partly by addition of solutes to cells. The latter is the major mechanism in *R. cancrivora*. Muscle inulin space (extracellular space) is constant in salinities between F.W. and 80 % S.W., and total muscle water content declines only 6 % over the same range (Table 3). Thus, muscle cell volume decreased only 7 % in the face of a nearly three-fold increase in plasma osmotic concentration.

Since the osmotic concentration inside muscle cells must equal that of the plasma, the contributions of the various solutes to intracellular osmotic concentration can be estimated (Fig. 2). Urea accounts for about 60 % of the total intracellular osmotic concentration change between F.W. and 80 % S.W. The sum of the concentrations of sodium, potassium and chloride ions accounts for an additional 10 % of the intracellular osmotic concentration change.

The values for α-amino nitrogen indicate that an increase in free amino acid concentration within the cell also contributes to the increase in intracellular osmotic concentration. The amount of intracellular α-amino nitrogen increases almost threefold between F.W. and 80 % S.W., an amount equivalent to about 95 mM free amino acids per kg. cell water. This increase accounts for almost 20 % of the osmotic concentration change. Results of quantitative analyses for specific amino acids were variable, but indicate that asparagine and/or glycine, β-alanine and carnosine may be the principal amino acids that change concentration in different salinities.

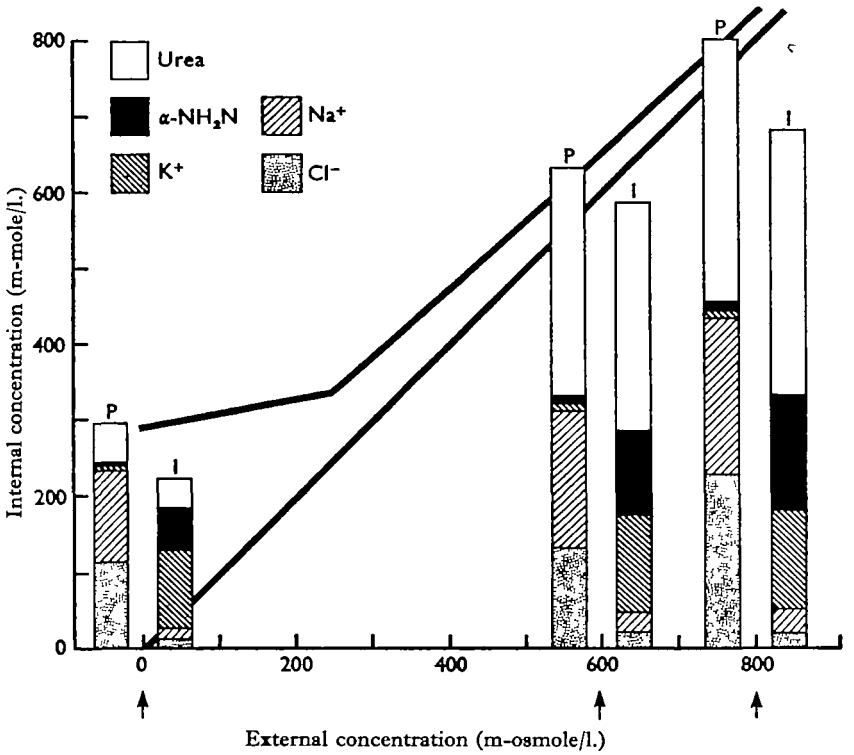

Fig. 2. Composition of plasma (P) and intracellular fluid (I) in skeletal muscles of *R. cancrivora* acclimatized to fresh water, 60 % and 80 % sea water. Upper continuous line, plasma osmotic concentration (from Gordon *et al.*, 1961); lower continuous line, equality between internal and external concentrations. Left-hand bar in each pair represents plasma concentrations; right-hand bar represents calculated intracellular concentrations. Arrows along abscissa mark acclimatization concentrations.

Approximately 10 % of the change in intracellular osmotic concentration remains not accounted for. The responsible solute may be part of the non-protein nitrogen fraction, other than urea and α-amino nitrogen. This can be seen from the intracellular concentration values for these fractions (Table 4). Urea nitrogen and α-amino nitrogen account for only 36 % of intracellular non-protein nitrogen in F.W., 79 and 71 % in 60 and 80 % S.W. respectively. These ratios are close to the values that would be expected if there were another, unidentified non-protein nitrogen fraction present, the concentration of which varies in proportion to changes in tissue hydration. This unidentified fraction could provide a large part of the change in intracellular osmotic

concentration which occurs between F.W. and 80 % S.W. and is not accounted for here. It also might account for the difference between plasma osmotic concentrations and calculated intracellular osmotic concentrations in different salinities (Fig. 2).

Intracellular changes in the muscles of *R. cancrivora* during osmoregulation can be compared to similar changes that occur in the euryhaline toad, *Bufo viridis* (Gordon, 1965). There are, however, quantitative differences between the two species in terms of the relative proportions of the various solutes used. Intracellular non-protein nitrogen levels in the frog are higher in given salinities than those found in the toad. In the frog, intracellular concentrations of urea are more than double those for free amino acid concentrations while these concentrations are nearly equal in the toad. The total intracellular concentration of free amino acids (about 150 mM/kg. cell water) in frogs in 80 % S.W. is about 3/4 the concentration found in the muscles of toads adapted to 50 % S.W. Finally, the toad does not appear to possess the unidentified, osmotically active, intracellular non-protein nitrogen fraction found in the frog.

SUMMARY

1. Total rates of urea loss from adult euryhaline crab-eating frogs (*Rana cancrivora*) adapted to various environmental salinities between fresh water and 80 % sea water increase as salinity increases above 40 % sea water. Oxygen consumption is constant in rate in all salinities studied.

2. The presence of urea in the Ringer solution bathing isolated pieces of skin of frogs adapted to 60 % sea water increases both the electrical potential and the inwardly directed short-circuit current across the skin.

3. In skeletal muscle cells addition of intracellular solutes maintains tissue hydration in the face of large increases in plasma osmotic concentration in high-salinity media. Changes in the intracellular urea and free amino acid concentrations are primarily responsible for increases in intracellular osmotic concentration.

4. Some implications of these observations are discussed and comparisons made with the euryhaline green toad, *Bufo viridis*.

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