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Water content, body weight and acid mucopolysaccharides, hyaluronidase and β-glucuronidase in response to aestivation in Australian desert frogs

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Abstract

This study investigates the effects of aestivation on body water content, body mass, acid mucopolysaccharide (AMPS) and some of its degrading enzymes in different tissues for some Australian desert frogs. The AMPS component of the liver, kidney, skin and cocoon alter during aestivation to help retain water, which is unchanged in most tissues of all frog species, and to protect the frogs from desiccation during extended periods of aestivation. Hepatic AMPS was unaltered in *Cyclorana maini*, *C. platycephala* and *Neobatrachus sutor* but increased significantly after 2 months of aestivation in *C. australis.* The level of AMPS in the kidney was elevated in all four frog species after 5 months of aestivation. Skin AMPS content in the skin of awake frogs decreases with aestivation period and increases in the cocoon. AMPS in the cocoon probably works as a cement between the cocoons' layers and its physical presence presumably contributes to preventing water flux. Changes in AMPS content in different tissues were accompanied by significant changes in both hyaluronidase and β-glucuronidase activities, which play an important role in AMPS metabolism. Alcian blue staining of control and digested skin of *C. australis* and *C. platycephala* with testicular hyaluronidase indicated the presence of AMPS, concentrated in a thin layer (called ground substance, GS) located between stratum compactum and stratum spongiosum, and acid mucin concentrated in the mucous glands and in a 'tubular' structure which could be observed in the epidermal layer. Hyaluronidase digestion of the cocoon slightly changed the Alcian Blue colour, suggesting the presence of a large amount of acid mucin similar to that found in the skin mucous gland. The results of this study present data for the redistribution of AMPS, which may help in reducing water loss across the cocoon and reabsorption of water in the kidney during aestivation. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Hibernating and aestivating animals need to maintain vital physiological processes during long periods of torpor and inanition. Water is considered one of the most vital substances required by these animals for their survival during the long dormancy periods of their life. Vertebrates and invertebrates use a variety of physiological and behavioural strategies to conserve water during extended periods of aestivation (Shylaja and Alexander, 1974; Appleton et al., 1979; Jørgensen, 1997). Frogs can minimise water loss by selecting favourable microclimates, secreting a lipid-like

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substance onto the skin, or shedding multiple layers of dead epidermis to produce a cocoon (Lee and Mercer 1967; Shoemaker et al., 1992; Davies and Withers, 1993; Withers, 1995). Western Australian desert frogs of the genera *Cyclorana* and *Neobatrachus* produce cocoons covering all parts of the body except for the narial openings. Little information is available on the composition of the mucus that has been observed between the skin and the cocoon, and between the layers of dead epidermis that form the cocoon (Damstra, 1983; McClanahan et al., 1983; Ruibal and Hillman, 1981; Withers, 1995), which plays an important role in reducing water loss in aestivating amphibians (Lillywhite and Licht, 1975; Christian and Parry, 1997). Mucopolysaccharide (MPS) has been found between the skin and the cocoon in *Cyclorana australis* and *Cyclorana platycephala* (Elkan, 1968; Van Beurden, 1984) and is considered to be one of the most important amphibian defences against desiccation (Elkan, 1968, 1976). MPS is also concentrated in the ground substance (GS) layer of the skin and may play an important role in binding water (Hvidberg, 1960; Block and Bettelheim, 1970). Lipson and Silbert (1968) and Wiederhielm et al. (1976) reported dermatan sulfate, chondroitin sulfate and hyaluronic acid as the only MPS in adult frog dorsal skin. MPS has also been shown to vary in concentration in other body tissues of hibernating animals, including the blood, kidney and liver (Zimny, 1973; Krestinskaya, 1964; Kupchella, 1968; Kupchella and Jacques, 1970; Kupchella et al., 1977; Patankar and Patil 1983). It increased in both blood of hibernating animals for the maintenance of haemofluidity during dormancy, and kidney to enhance water reabsorption. The objective of this study was to determine whether levels of MPS change in the skin, liver and kidney of aestivating desert frogs and have the same functions as in hibernating animals (Kupchella, 1968; Kupchella et al. 1977).

2. Materials and methods

2.1. Frogs

Three species of Western Australian desert burrowing frogs (*Cyclorana maini, Cyclorana platycephala* and *Neobatrachus sutor*) were collected in December 1999 following heavy rain approximately 70 km south of Newman, Western Australia (23°21′S, 119°43′E). Frogs were awake on the surface and males were calling when collected.

2.2. Experimental design

Frogs were sorted into their respective species and randomly assigned to an ‘active’ and an ‘aestivating’ group. They were weighed whilst awake to ± 0.0001 g (Sartorius balance, H51) and then again following disinterment. The awake group, was processed within the first week of capture and before any attempt had been made by frogs to aestivate. *Cyclorana maini, C. platycephala and N. sutor* were induced to aestivate by placing them individually in 500-ml plastic containers half-filled with moist soil collected at their site of capture and then placed in a constant room temperature maintained at 20 °C with no access to free water. *Cyclorana australis* were induced to aestivate by placing them individually in plastic containers without soil and at room temperature in a dark cupboard (23 °C) for 2 months. Aestivation was considered to commence as soon as the frogs burrowed below the soil surface or for *C. australis* the first layer of shed skin became obvious. Frogs were disinterred at intervals of 2, 5 and 7 months, weighed, killed by pithing, and then tissues processed. All changes were then expressed relative to values recorded in awake frogs.

2.3. Tissue excision

The cocoon was removed immediately before pithing. Liver, kidney and samples of dorsal and ventral skin were taken from the dead specimen. Each tissue sample was divided into three parts; the first was kept in formal saline solution for histochemical studies, the second was weighed to ± 0.1 mg for determination of total water content, and the third was homogenised for biochemical analysis. Tissues to be homogenised were stored frozen at -20 °C until analysed. Accurately weighed portions were homogenised in Tris–chloride buffer (pH 7.5) in an ice bath using a glass homogeniser.

2.4. Biochemical analyses

Total AMPS was measured for liver, kidney, dorsal and ventral skin, and cocoon samples according to the method of Edstrom (1969) using a colorimetric method for the estimation of small

amounts (0.5–5g) of acidic polysaccharides using the shift in the visible absorption spectrum of carbocyanine dye when it is bound to a polyanion.

Activity of β-glucuronidase (EC 3.2.1.31) in the liver, kidney, dorsal and ventral skin and cocoon was determined with Sigma diagnostics kit (Catalogue No. 325-A) using a modified method of Fishman et al. (1967) based on the cleavage of phenolphthalein glucuronic acid by β-glucuronidase, producing phenolphthalein which is then determined colorimetrically at 550 nm. One modified Sigma unit of β-glucuronidase activity will liberate 1 μg of phenolphthalein from phenolphthalein glucuronic acid per hour at 56 °C.

Activity of hyaluronidase (EC 3.2.1.30) in the liver, kidney, dorsal and ventral skin, and cocoon was determined using the method of Bonner and Cante (1966). It is based on the quantification of *N*-acetylglucosamine end groups liberated from hyaluronic acid (HYA). The amount of *N*-acetylglucosamine released by 0.1 ml of 0.05 mg/ml bovine testicular hyaluronidase (330 USP unit/mg).

2.5. Determination of water content

Tissues were weighed wet then dried in an oven at 60 °C for 2 days and reweighed.

2.6. Microscopical examination

Small portions of dorsal skin and cocoon from *C. australis* and *C. platycephala* were placed in 10% formal saline solution, embedded in wax and 6-μm-thick histological sections were prepared. Two slides were prepared of each specimen. All sections were dewaxed and one slide was digested using testicular hyaluronidase in phosphate buffer (pH 6.7) for 3 h at 37 °C, according to the method of Pearse (1953). The other slide was treated with buffer only as a control. All sections were stained with Alcian blue (pH 2.5) for 5 min, rinsed in alcohol, cleared in xylene, and mounted.

2.7. Analysis of data

Differences between *C. maini*, *C. platycephala* and *N. sutor* over time were analysed using one-way ANOVA followed by post-hoc Tukey tests. Student's *t*-test was used to determine differences between means for *C. australis* for which only two time intervals were available. Non-parametric tests (Mann–Whitney) were used when standard deviations of the samples differed significantly.

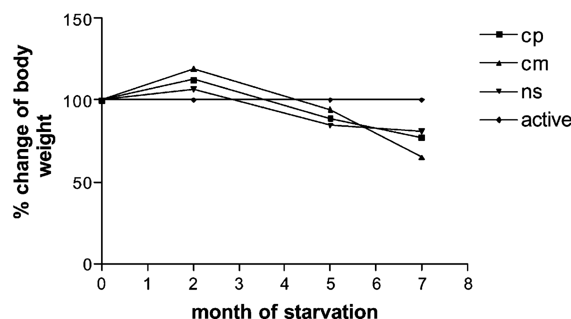

Fig. 1. Percent change of body weight during aestivation in Australian desert frogs.

3. Results

3.1. Body mass

The body mass of all species increased significantly during the first 2 months of aestivation and then fell below awake levels at 5 and 7 months of aestivation. These results indicate water absorption during the first 2 months of aestivation, and water loss after this period (Fig. 1).

3.2. Body water content

Although aestivating desert frogs had no access to free water the water content of skin, liver and kidney increased for all species during aestivation. Hepatic, renal and skin water content were 65%, 80% and 72% in awake *C. platycephala* (Table 1) whereas these contents slightly increased to 70.5%, 82.9% and 76% after 5 months of aestivation, respectively. Tissue water content of the liver and kidney of *N. sutor* (Table 1) showed insignificant changes. In contrast, for *C. maini* the hepatic water content dropped noticeably after 7 months of aestivation ($P<0.001$). The skin of *C. maini* and *N. sutor* absorbed water during the first 2 months of aestivation, and therefore the water content increased by 13.8% and 7.1%, respectively. The water content of dorsal (DS) and ventral skin (VS) and the kidney of *C. australis* did not change significantly compared with awake frogs.

3.3. Acid mucopolysaccharide content

Hepatic AMPS slightly changed when compared with the awake frogs during the study period for

Table 1
Percent water content of tissues of different Australian desert frog during different time intervals of aestivation

Animal	Organ	Active	*N*	2 month	*N*	5 month	*N*	7 month	*n*
Cyclorana platycephala	Liver	65.03±3.82	5	65.5±6.3	5	70.5±2.68	4	66.3±4.69	5
	Kidney	80.2±1.03	4	80.2±1.87	5	82.9±2.65	4	76.3±5.27	5
	Dorsal skin	70.8±2.01	5	74.8±1.76	5	76.2±3.41	4	71.8±5.78	5
	Ventral skin	73.8±3.63	5	75.6±1.34	5	76.4±4.56	4	70.2±4.8	5
Cyclorana maini	Liver	70.5±4.52	5	66.7±4.02	4	68.1±2.25	5	52***±4.9	4
	Kidney	78.9±1.48	6	82.2±9.17	4	81.2±5.04	5	82.6±1.93	4
	Dorsal skin	69.3±4.25	6	74.2±1.6	4	69.8±3.43	5	64.7±3.79	4
	Ventral skin	69.3±4.25	6	76.1*±3.31	5	78.5±4.15	4	66.6±2.8	4
Cyclorana australis	Liver	73.5±5.3	11	82.02*±8.9	7	–	–	–	–
	Kidney	77.4±9.6	10	75±6.5	7	–	–	–	–
	Dorsal skin	68.1±7.9	11	76.6±9.15	7	–	–	–	–
	Ventral skin	69.2±12.1	10	73.9±5.95	7	–	–	–	–
Neobatrachus sutor	Liver	60.1±5.7	6	60.1±5.9	6	50.2±6.09	5	58.2±5.4	4
	Kidney	78.3±4.4	6	75.1±3.2	6	76.1±1.67	5	78.3±5.5	4
	dorsal skin	68.6±2.8	6	78.1**±4.8	6	75.9*±3.98	5	71±3.63	4
	Ventral skin	68.6±2.8	6	78.3***±3.51	6	75.5*±2.8	5	71.9±4	4

Values are mean±standard deviation, with sample size (n). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

all species, except for *C. australis* where hepatic AMPS (Table 2) increased significantly after 2 months of aestivation ($P<0.05$). Renal AMPS of *C. australis, C. platycephala, C. maini* and *N. sutor* (Table 2) increased significantly ($P<0.01$), reaching a peak at 5 months of aestivation compared with awake animals (Table 2).

Total AMPS content for DS, of *C. maini,* decreased reaching their lowest values at 5 months (118.0±24 units) and for VS at 7 months (74±9 units) of aestivation compared with awake animals. Cocoon content of AMPS increased significantly, reaching its maximum value after 5 months of aestivation (44.9%). Skin and cocoon contents of

Table 2
Total acid mucopolysaccharide content (as mg /gm dry tissue) of tissues for Australian desert frogs at different time intervals of aestivation

Animal	Organ	Active	*N*	2 month	*N*	5 month	*N*	7 month	*n*
Cyclorana platycephala	Liver	158.5±31.8	5	196.21±31.2	5	197.9±26.6	4	190.4±31.02	4
	Kidney	366.9±79.5	4	432.7±39.8	5	690.7***±116.2	4	496.4±42.7	4
	Dorsal skin	248.8±50.9	5	176*±12.7	5	170.3*±9.8	4	113.9***±31.3	4
	Ventral skin	145.3±26.6	4	72.4***±7.5	5	95.8**±15.6	4	93.1**±13.1	4
	Cocoon	–	–	215.7±15	5	240*±9.4	4	273.3***±4.8	4
Cyclorana maini	Liver	92.9±22.1	5	98.7±12.9	4	124.3±13.7	4	84±10.1	4
	Kidney	271±43.9	6	289.6±51.9	4	582.2***±67.1	4	496.3***±104.8	4
	Dorsal skin	196.7±14	6	159.2*±9	4	117.6***±24	4	144.3***±14.7	4
	Ventral skin	202.5±47.5	6	163.3±26.2	4	104**±37.2	4	73.9***±8.5	4
	Cocoon	–	–	214.3±11.5	4	310.59±32.6	4	292.67±6.2	4
Cyclorana australis	Liver	151.9±25.7	11	208.8±49.7	7	–	–	–	–
	Kidney	123.6±20.3	11	230.5***±66.5	7	–	–	–	–
	Dorsal skin	68.3±21.1	11	47.7±16.04	7	–	–	–	–
	Ventral skin	103.1±51.3	11	65.6±16.1	7	–	–	–	–
Neobatrachus sutor	Liver	124.7±15.1	6	103.8±14.7	6	121.4±9.9	4	124.7±20.8	4
	Kidney	229.7±56.6	5	220.1±40.3	6	406.4***±24	4	296.5±51.7	4
	Dorsal skin	182.5±29.4	6	217.5±57.3	6	92.6***±16.5	4	159.4±33.2	4
	Ventral skin	272.1±37.1	6	199.5**±24.6	6	92.6***±8.7	4	109.7***±15.9	4
	Cocoon	–	–	161.9±10.9	6	216***±5	4	233.1***±7.03	4

Values are mean±standard deviation, with sample size (n). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 3
β-Glucuronidase activity (units/mg tissue protein) in the tissues of different Australian desert frogs during different time intervals of aestivation

Animal	Organ	Active	N	2 month	n	5 month	N	7 month	n
Cyclorana platycephala	Liver	45.5±2.4	5	52.2±3.9	5	32.08***±5.5	4	13.9***±2.5	4
	Kidney	25.9±6.6	5	29.3±7.4	5	35.3±2.04	4	62.3***±8.3	4
	Dorsal skin	8±1.6	5	11.9**±1.8	5	3.5**±0.31	4	2.4***±0.36	4
	Ventral skin	13.2±2.7	5	15.5±1.3	5	8.2**±1.6	4	10.05±1.01	4
	Cocoon	–	–	11.3±1.4	5	0.93***±0.5	4	1.06***±0.30	4
Cyclorana maini	Liver	95.8±15.6	6	119.03*±7.9	4	28.5***±2.9	4	12.7***±1.02	4
	Kidney	50.6±7.9	6	129.7***±9.5	4	86.4***±7.7	4	63.9±15.2	4
	Dorsal skin	4.3±1.5	5	7.2**±0.79	4	2.3*±0.45	4	2.4*±0.26	4
	Ventral skin	15.3±3.9	5	14.8±1.8	4	6.6***±0.65	4	4.4***±0.73	4
	Cocoon	–	–	24.9±9.3	4	1.04***±0.37	4	4.4**±0.58	4
Cyclorana australis	Liver	76.72±6.81	10	107.59***±2.79	7	–	–	–	–
	Kidney	48.99±15.64	10	140.52***±5.88	7	–	–	–	–
	Dorsal skin	34.81±4.76	11	50.383***±6.49	7	–	–	–	–
	Ventral skin	23.68±3.89	11	43.28***±6.71	7	–	–	–	–
Neobatrachus sutor	Liver	67.1±8.2	6	65.7±6.9	6	3.3***±0.39	4	5.7***±0.39	4
	Kidney	26.3±4.8	6	68.1***±6.4	6	19.5±0.78	4	17.6*±2.4	4
	Dorsal skin	26.2±2.7	6	11.1***±0.87	6	2.5***±0.57	4	3.3***±0.44	4
	Ventral skin	9.7±2.6	6	8.2±1.4	6	4.7**±1.4	4	4.06***±0.40	4
	Cocoon	–	–	9.8±1	6	7.09**±0.99	4	1.9***±0.36	4

Values are mean±standard deviation, with sample size (n). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

AMPS of the other desert frog species were found to be changed in a similar way during aestivation.

3.4. Activity of β-glucuronidase

β-Glucuronidase activity in the various tissues for Western Australia desert frogs is summarised in Table 3. Hepatic β-glucuronidase activity in aestivating *C. maini* increased significantly by 24.1% to 119±7.9 U/mg tissue protein ($P<0.05$) after 2 months of aestivation; it then decreased significantly ($P<0.01$) after 5 and 7 months of aestivation to 28.5±2.9 and 12.7±1.0 units/mg tissue protein respectively. Similar patterns of change were also observed for hepatic β-glucuronidase in *C. platycephala* and *N. sutor*.

Renal β-glucuronidase activity of *C. maini* and *C. platycephala* showed a significant increase ($F_{3,14}=51.57$ and $F_{3,14}=26.2$, respectively) after a prolonged period of aestivation. In contrast, renal β-glucuronidase activity of *N. sutor* decreased significantly to 19.5±0.8 units/mg tissue protein (by 26%) after 5 months, and to 17.6±2.4 units/mg tissue protein (by 33%) after 7 months of aestivation.

β-Glucuronidase in kidney of *C. australis* increased from 49±16 to 140.5±5.9 units/mg tissue protein. These results reflect that β-glucuronidase activity in the liver was decreased whereas its activity in kidney increased throughout aestivation. β-Glucuronidase activity in DS and VS changed significantly in *C. maini*, *C. platycephala* and *N sutor* during aestivation. The β-glucuronidase activity of DS increased during the first 2 months of aestivation then it decreased for the remainder of the period. For example, in *C. platycephala* the DS β-glucuronidase activity increased from 8.02±1.6 to 11.9±1.8 ($P<0.01$) then it decreased to 3.5±0.31and 2.4±0.36 after 5 and 7 months, respectively. β-Glucuronidase activity in VS decreased progressively in all species except for *C. australis*. Activity of β-glucuronidase of the cocoon of *C. platycephala, C. maini* and *N. sutor* decreased from 11.3±1.4, 24.9±9.3 and 9.8±1 to 0.93±0.5, 1.04±.37 and 7.09±0.99, respectively, after 5 months of aestivation. These activities of the cocoon are similar or even exceed those of skin.

There was a significant increase in β-glucuronidase activity in the DS and VS of cocooned *C. australis* after 7 month of aestivation.

3.5. Activity of hyaluronidase

Summaries of hyaluronidase activity for all species when awake and aestivating are shown in

Table 4
Hyaluronidase activity (units/g tissue protein) in the tissues of different Australian desert frogs during different time intervals of aestivation

Animal	Organ	Active	*N*	2 month	*N*	5 month	N	7 month	N
Cyclorana platycephala	Liver	98.7±7.5	4	126.9±15.5	4	113.6±16.6	4	147.8**±14.8	4
	Kidney	85.5±16.1	4	171.1*±36.3	4	168.6*±32.2	4	259.2***±38.04	4
	Dorsal skin	4.7±0.92	4	17.3**±1.6	4	42.7***±6.4	4	29.9***±3.9	4
	Ventral skin	9.2±3.1	4	17.8**±2.9	4	30.6***±3.2	4	22.6***±1.2	4
	Cocoon	–	–	18.6±2.0	4	24.9*±4.02	4	23.1±2.4	4
Cyclorana maini	Liver	100.7±7.2	4	184.7**±28.9	4	141.1±9.9	4	339.9***±51.1	4
	Kidney	87.8±8.7	4	199.2***±30.5	4	211.5***±21.8	4	359.3***±19.9	4
	Dorsal skin	6.2±0.59	4	28.1***±2.7	4	28.6***±3.9	4	33.1***±5.9	4
	Ventral skin	7.7±1.5	4	29.2**±6.5	4	54.7***±10.8	4	49.3***±4.08	4
	Cocoon	–	–	26.8±7.4	4	24.6±0.701	4	18.8±1.8	4
Cyclorana australis	Liver	98.589±14.652	4	157.34***±7.639	4	–	–	–	–
	Kidney	146.12±15.09	4	136.85±19.727	4	–	–	–	–
	Dorsal skin	146.22±32.626	4	330.65***±40.817	4	–	–	–	–
	Ventral skin	39.917±8.48	4	80.907**±12.387	4	–	–	–	–
Neobatrachus sutor	Liver	141.2±22.4	4	243.9***±21.7	4	322.05***±39.4	4	290.7***±10.9	4
	Kidney	105.6±20.3	4	219.2***±34.2	4	259.5***±15.1	4	318.5***±34.1	4
	Dorsal skin	18.8±3.6	4	35.3**±6.1	4	49.4***±4.2	4	50.2***±6.4	4
	Ventral skin	21.6±8.08	4	35.3±3.4	4	39.4±11.3	4	42.8*±8.9	4
	Cocoon	–	–	45.3±7.7	4	68.3*±14.06	4	72.5*±8.6	4

Values are mean±standard deviation, with sample size (*n*). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table 4. Hyaluronidase activity increased significantly in most tissues in all species during aestivation. Hyaluronidase activity in *C. maini* reached its maximum levels in the liver after 2 months (184.7±28.9 units/g tissue protein) and the kidney (211.5±21.8 units/g tissue protein) after 5 months. Its increase in DS and ventral skin exceeds 100% of its amount in awake frogs' skin that was very low. Hyaluronidase activity in the DS increased by 435% and in VS by 604% after 7 months of aestivation for *C. maini*. The hyaluronidase activity in the cocoons of *C. maini* remained unchanged during prolonged periods of aestivation.

In *N. sutor,* hyaluronidase activity significantly increased in the liver ($F_{3,12}=37.6$, $P<0.01$), kidney ($F_{3,12}=43$ $P<0.01$), DS ($F_{3,12}=31.8$, $P<0.01$), VS ($F_{3,12}=4.8$, $P=0.02$) and cocoon ($F_{2,9}=7.8$, $P=0.01$) during aestivation. Hyaluronidase activity in *C. platycephala* increased in all tissues, reaching its maximum in the liver (147.8±14.8 units/g tissue protein) and kidney (259.2±38 units/g tissue protein) after 7 months of aestivation, which amounted to +49.7% and +203.2%, respectively. Hyaluronidase activity increased to maximum levels in the DS (42.7±6.4 units/g tissue protein), VS (30.6±3.2 units/g tissue protein) and cocoon (24.9±4 units/g tissue protein) after 5 months of aestivation.

Hyaluronidase activity in the DS and VS of cocooned *C. australis* increased by 126% and 103%, respectively, after 2 months of aestivation compared with awake animals.

3.6. Skin morphology

By staining skin sections of *C. australis* and *C. platycephala* with Alcian Blue (pH2.5), we identified (Fig. 2A,B) the stratum corneum, epithelium (E), stratum spongiosum (S) which incorporated various glands in a network of collagen and elastic fibres, smooth muscle fibres, chromatophores, nerves, and its network of blood capillaries, ground substance (GS) and the stratum compactum (C).

There are two parts of the dorsal skin of both awake and cocooned *C. australis* and *C. platycephala* that are strongly stained with Alcian Blue. The first part is the mucus gland indicating acid mucin. The second is the ground substance layer, indicating AMPS.

There is a network of 'tubular' structures in the lower part of the epidermal layer which was easily observed in dorsal skin of awake and cocooned *C. australis* and cocooned *C. platycephala,* but was difficult to detect in awake *C. platycephala*. This network of 'tubules' is positively stained with Alcian Blue. The first stained layer of the awake

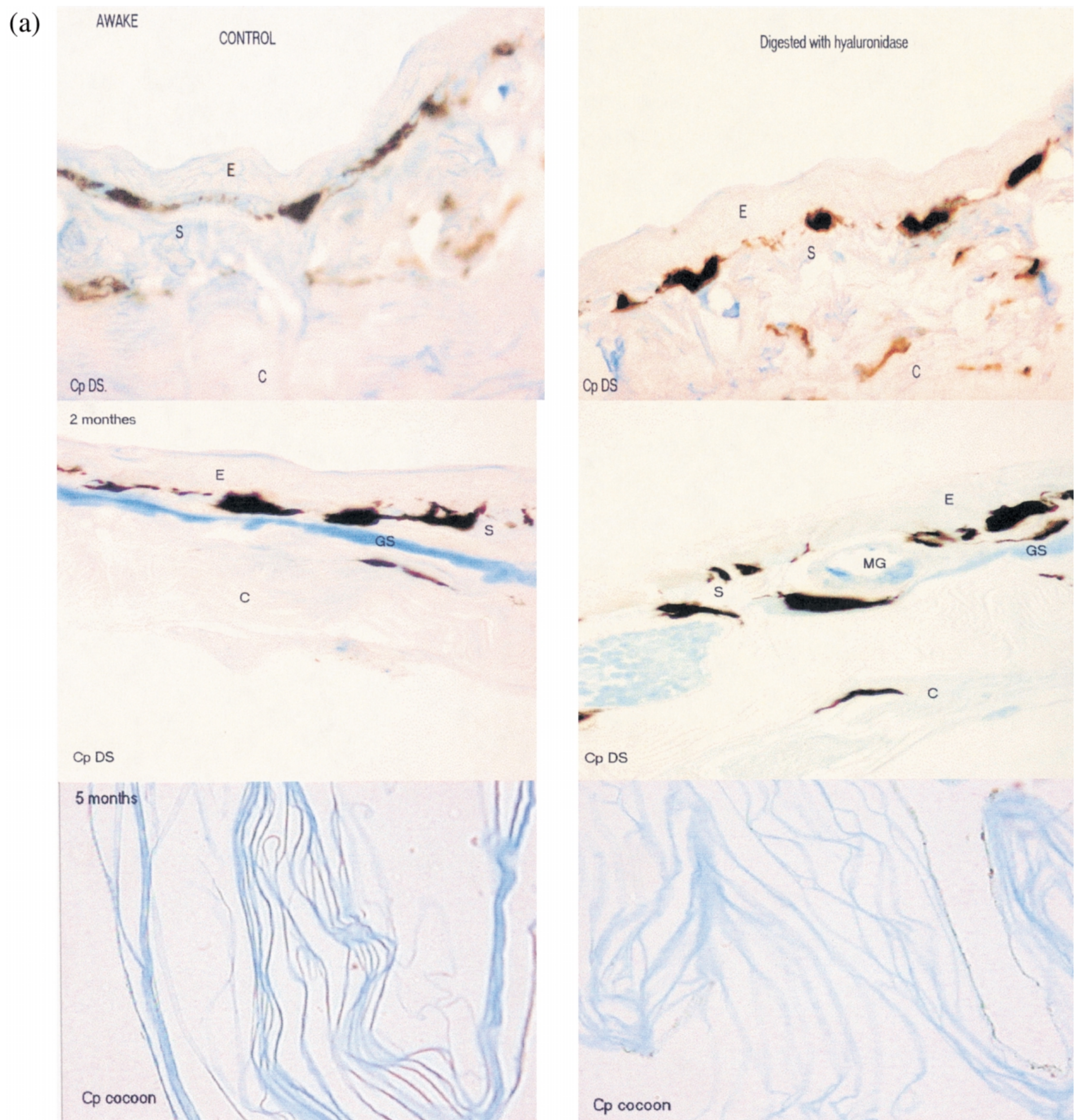

Fig. 2. Sections of dorsal skin (DS) and cocoon, control and digested (with hyaluronidase), of active and cocooned: (A) *C. platycephala* (Cp) and (B) *C. australis* (Ca). (E) epidermis, (MG) mucus gland, (GS) ground substance, (S) stratum spongiosum, (C) stratum compactum.

dorsal skin of *C. australis* appears ready to separate from the skin while this and the second layer which is stained with Alcian Blue (to form the first layer of the separated cocoon when the animals enter aestivation). There are some layers of the cocoon that are still attached to the dorsal skin of cocooned animals and are stained with Alcian Blue. The Alcian Blue could be seen in different parts of dorsal skin in both awake and cocooned animals indicating the presence of AMPS in different parts of skin.

In dorsal skin and cocoon of *C. australis* and *Cyclorana platycephala* digested with testicular hyaluronidase, which is known to digest HYA and

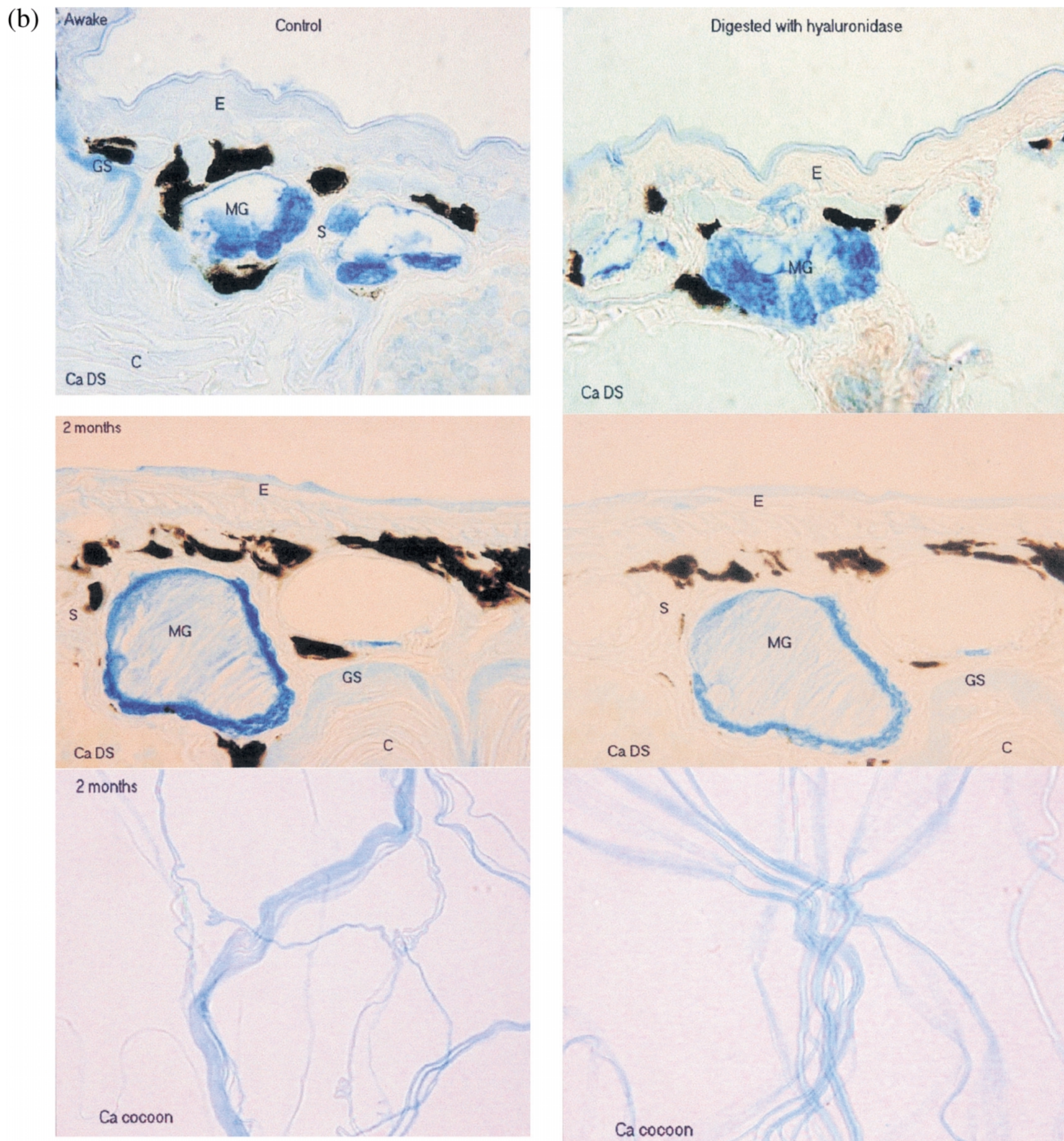

Fig. 2 (Continued).

chondroitin sulfate A and C (hence remove them from the sections) but not acid mucine, we can identify:

1. the glands become paler in colour but are still stained with a strong blue colour, especially their edges, indicating the presence of some HYA and/or chondroitin sulfate;
2. the ground substance becomes very pale in colour indicating it is rich in HYA and/or chondroitin sulfate A and C;
3. the 'tubular' structures that can be seen in the epidermal layer are still stained blue although

they become paler, indicating they contain acid mucin and very little HYA and/or chondroitin sulfate A and C. This observation suggests that this 'tubular' network carries gland secretions;

4. the cocoon layers are still strongly stained with blue colour, indicating the presence of a large amount of acid mucins rather than HYA or chondroitin sulfate A and C in or around them; and
5. other parts of the dorsal skin become pale in colour indicating the presence of HYA and/or chondroitin sulfate A and C.

4. Discussion

MPS is thought to be one of the most important materials that protect amphibians against desiccation. MPS is abundant in the amphibian skin in a layer called ground substance (Elkan, 1968), the main components of which are neutral and acid mucopolysaccharides, sulfated or non-sulfated. Among these, HYA plays a dominant part because of its water–binding capacity (Meyer, 1947; Rogers, 1961). Our morphological observations indicate the presence of the ground substance in both awake and aestivating *C. australis,* but it is in low levels in awake *C. platycephala,* only developing after 2 months of aestivation. The morphology of the skin when digested with testicular hyaluronidase indicates the presence of HYA and/or chondroitin sulfate A and C in the ground substance of both awake and aestivating frogs. The presence of HYA and/or chondroitin sulfate A and C in the skin may play a role in tissue water-binding and the percent water content generally does not decrease in the tissues described in this study that contain ground substance. Biochemical analysis of both skin and cocoon showed that the amount of AMPS in skin decreased during prolonged aestivation while the amount in the cocoon exceeds that in skin and significantly increased with aestivation. These results indicate that AMPS in the cocoon must come from skin, and support the suggestion of Withers (1998) that the hygroscopic nature of the cocoon could involve MPS because of their ability to bind water. In this study, both hyaluronidase and β-glucuronidase were detected in Australian desert frog skin. Hyaluronidase was found to increase significantly in all tissues during aestivation. These results contrast with those of Lipson et al. (1971) who reported that adult *Rana catesbiana,* dorsal skin was devoid of hyaluronidase activity which is present in tadpole skin and is involved in removing glycosaminoglycans during metamorphosis. The presence of hyaluronidase in aestivating frogs suggests a possible role that hyaluronidase may play in removing MPS during the separation of the presumptive cocoon layer from the skin, to form the multi-layered 'stratum corneum' cocoon (Lee and Mercer, 1967; Ruibal and Hillman, 1981; Van Beurden, 1982, 1984; Davies and Withers, 1993; Withers, 1995).

Body mass generally increased during the first 2 months of aestivation and then decreased, indicating the ability of these frogs to absorb water from the initially-moist soil and store it in the body for use during aestivation. This absorption of water from the moist soil presumably continues until the formation of a thick cocoon which will render the skin essentially impermeable to water movements, and the soil becomes too dry for water absorption (Reno et al., 1972; McClanahan et al. 1976). The presence of large quantities of AMPS in the cocoon suggests this compound is linked to the 'water-proofing' of the cocoon as mentioned by Duellman and Trueb (1986).

The histological preparation of both control and digested sections of the cocoon indicate the presence of large amounts of acid mucin that is probably surrounding the cocoon layers. The same material could be seen in the mucous gland of both awake and aestivating animals' skin and in the 'tubular' network in the epidermal layers. The fact that mucus exists between the cocoon layers, which seems to be secreted by the mucous glands through those 'tubular' structures in the epidermis, has appeared to be linked with a role in reducing water loss (Lillywhite and Licht, 1975; Ruibal and Hillman, 1981; Damstra, 1983; Withers, 1995; Christian and Parry, 1997). MPS has been implicated in cellular adhesions (Overton, 1969; Khan and Overton, 1969, 1970; Pessac and Defendi 1972) and its presence in the cocoon and skin may play the same role in addition to its functions in water-binding and protection against desiccation (Hvidberg, 1960; Elkan, 1976; Wiederhielm et al., 1976). Many investigators have demonstrated the importance of extracellular materials in the cellular interactions that are necessary for normal morphogenesis and differentiation and MPS has been implicated in these interactions (Kallman and Grobstein, 1966; Slavkin et al., 1969; Bernfield

and Wessells, 1970; Bernfield and Banerjee, 1972; Bernfield et al., 1972).

Levels of hepatic AMPS of *C. australis, C. maini* and *N. sutor* were found to decrease in response to aestivation whilst in *C. platycephala* they increased. The change in the AMPS content of the liver was accompanied by an increase in the activity of hyaluronidase. There may be a correlation between AMPS in the liver and its level in plasma and hence other tissues of the body especially with the increase of AMPS in the kidney (Ginetsinskii, 1959; Krestinskaya, 1964). Our results indicate an increase of AMPS in the kidney of all frog species accompanied by an elevation in MPS-degrading enzymes, especially hyaluronidase, which gives an indication of the decrease of HYA and chondroitin sulfate and the elevation of other AMPS. These results are similar to those obtained by Zimny (1973) for the hibernating 13-lined ground squirrel and Patankar and Patil (1983) for hibernating bats. It was suggested that the increase of this anionic material may be responsible for maintaining water and electrolyte balance by acting as a resin for the exchange of cations between the capillary and urinary spaces, attracting water and concentrating protein, since MPS contains sialic acid which has the capability to concentrate protein. This too may be a function of the increased acid MPS in the glomerular basement membrane. The protein may then move to the urinary space and be imbibed by pinocytotic activity of the proximal convoluted tubules as was suggested by Zimny et al. (1971). However, AMPS has an important role in the action of antidiuretic hormones (ADH) in mammalian kidneys (Ginetsinskii, 1959, 1961). There is a correlation between the development of the hyaluronidase-hyaluronic acid system and reaction to antidiuretic hormone for water reabsorption (Natochin, 1960). Guinetzinsky (1958) suggested that HYA is the ultimate target for antidiuretic hormone playing an important role in increasing the rate of water reabsorption in the kidney tubules. According to this hypothesis antidiuretic hormone activates hyaluronidase, which depolymerises HYA and certain chondroitin sulfates leading to an alteration of the histostructure of the kidney favouring water reabsorption (Corbascio and Dong, 1966; Dicker and Eggleton, 1960, 1961; Ratner and Tomilina, 1966). This hypothesis may explain the significant increase of hyaluronidase in the kidney and in the other organs studied. It is well known that anurans that inhabit arid environments, including Australian desert frogs, can store large quantities of water in their urinary bladder (cf. Main and Bentley, 1964). The water content of the tissues was maintained constant under burrowing conditions. The water content and distribution amongst the various body compartments such as circulation, interstitial, intracellular and urinary system seems therefore to be the more strictly controlled variable.

In conclusion, mucopolysaccharide appears to be a vital and regulated biochemical compound in various tissues of aestivating frog, presumably playing an important role in controlling water flux in the different body tissues, especially the skin and kidneys.

References

Appleton, T.C., Newell, P.F., Machin, J., 1979. Ionic gradients within mantle-collar epithelial cells of the land snail *Otala lactea*. Cell Tissue Res. 199, 83–97.

Bernfield, M.R., Wessells, N.K., 1970. Intra and extracellular control of epithelial morphogenesis. In: Runner, M.N. (Ed.), Changing Synthesis in Development, Academic Press, New York pp. 195–249.

Bernfield, M.R., Banerjee, S.D., 1972. Acid mucopolysaccharide (glycosaminoglycan) at the epidermal–mesenchymal interface of mouse embryo salivary glands. J. Cell Biol. 52, 664–673.

Bernfield, M.R., Banerjee, S.D., Cohn, R.H., 1972. Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide protein (proteoglycan) at the epithelial surface. J. Cell Biol. 52, 674–689.

Block, A., Bettelheim, F.A., 1970. Water vapor sorption of hyaluronic acid. Biochem. Biophys. Acta 201, 69–75.

Bonner, W.M., Cante, E.Y., 1966. Colorimetric method for determination of serum hyaluronidase activity. Clin. Chim. Acta 13, 746–752.

Christian, K., Parry, D., 1997. Reduced rates of water loss and chemical properties of skin secretions of the frogs *Litoria caerulea* and *Cyclorana australia*. Aust. J. Zool. 45, 13–20.

Corbascio, A.N., Dong, L., 1966. The mechanism of action of antidiuretic hormone. Inv. Urol. 4, 267–272.

Damstra, K.S.J., 1983. The anuran skin gland duct — a significant avenue for evaporative water loss. Elect. Mic. Soc. South Africa Proc. 13, 73–74.

Davies, M., Withers, P.C., 1993. Morphology and physiology of the *Anura*. In: Glasby, C.J., Ross, G.J.B., Beesley, P.L. (Eds.), Fauna of Australia, Amphibia and Reptilia, Vol. 2A. Australian Government Printing Service, Canberra pp. 15–27.

Dicker, S.W., Eggleton, M.G., 1960. Hyaluronidase and antidiuretic activity in urine of man. J. Physiol. 154, 378–384.

Dicker, S.W., Eggleton, M.G., 1961. Renal excretion of hyaluronidase and calcium in man during the antidiuretic action of vasopressin and some analogs. J. Physiol. 157, 351–362.

Duellman, W.E., Trueb, L., 1986. Biology of Amphibians. McGraw-Hill, New York.

Edstrom, R.D., 1969. A colorimetric method for the determination of mucopolysaccharides and other acidic polymers. Anal. Biochem. 29, 421–432.

Elkan, E., 1976. Ground substance: an anuran defence against desiccation. In: Lofts, B. (Ed.), Physiology of Amphibia, Academic Press, New York pp. 101–110.

Elkan, E., 1968. Mucopolysaccharides in the anuran defence against desiccation. J. Zool. Lond. 155, 19–53.

Fishman, W.H., Kato, K., Anstiss, C.L., Green, S., 1967. Human serum β-glucuronidase, its measurement and some of its properties. Clin. Chim. Acta 15, 435.

Ginetsinskii, A.G., 1959. Evolution of the water excreting function of the kidney. Fiziol. Zh. Sssr. 45, 761.

Ginetsinskii, A.G., 1961. Role of hyaluronic structures in evolution of water-excretory function of the kidney. Zh. Obshch. Biol. 22, 1–9.

Guinetzinsky, A.G., 1958. Role of hyaluronidase in the reabsorption of water in renal tubules: the mechanism of action of the antidiuretic hormone. Nature (Lond.) 182, 1218–1219.

Hvidberg, E., 1960. Water-binding by connective tissue and the acid mucopolysaccharides of the ground substance. Acta Pharmacol. Toxicol. 17, 267–276.

Jørgensen, C.B., 1997. 200 years of amphibian water economy: from Robert Townson to the present. Biol. Rev. Camb. Phil. Soc. 72, 153–237.

Kallman, F., Grobstein, C., 1966. Localization of glucosamine-incorporating materials at epithelial surfaced during salivary epithelio–mesenchymal interaction. Dev. Biol. 14, 52–67.

Khan, T., Overton, J., 1969. Staining of intercellular material in reaggregating chick liver and cartilage cells. J. Exp. Zool. 171, 161–174.

Khan, T., Overton, J., 1970. Lanthanum staining of developing chick cartilage and reaggregating cartilage cells. J. Cell Biol. 44, 433–438.

Krestinskaya, T.V., 1964. Mucopolysaccharides in kidneys of vertebrates of various classes. Ark. Anat. Gisto. Embriol. 47, T629–T631.

Kupchella, C., 1968. The Effects of Induced Cold Torpor and Time of Year in *Chrysemys picta belli* and *Pseudemys scripta elegans*, Ph.D. Thesis. St. Bonaventure University, Univ. Microfilms, Ann Arbor, MI.

Kupchella, C.E., Jacques, F.A., 1970. The effects of induced cold torpor and time of year on the plasma acid mucopolysaccharide levels in *Pseudemys scripta* elegans and *Chrysemys picta* belli. Comp. Biochem. Physiol. 36, 657–668.

Kupchella, C.E., Heckman, J.R., Lega, R.D.and, Plamp, C.E., 1977. The influence of cold-torpor and water deprivation on plasma and kidney glycosaminoglycans in emydine turtles. Comp. Biochem. Physiol. A 56, 225–229.

Lee, A.K, Mercer, E.H., 1967. Cocoon surrounding desert-dwelling frogs. Science 157, 87–88.

Lillywhite, H.B., Licht, P., 1975. A comparative study of integumentary mucus secretions in amphibians. Comp. Biochem. Physiol. A 51, 937–941.

Lipson, M.J., Silbert, J.E., 1968. Glycosaminoglycans of adult frog back skin. Biochim. Biophys. Acta 158, 344–350.

Lipson, M.J., Cerskus, R.A., Silbert, J.E., 1971. Glycosaminoglycans and glycosaminoglycan-degrading enzyme of *Rana catesbeiana* back skin during late stages of metamorphosis. Dev. Biol. 25, 198–208.

Main, A.R., Bentley, P.J., 1964. Water relations of Australian burrowing frogs and tree frogs. Ecology 45, 379–382.

McClanahan, L.L., Shoemaker, V.H., Ruibal, R., 1976. Structure and function of the cocoon of a ceratophryd frog, Copeia, 179–185.

McClanahan, L.L., Ruibal, R., Shoemaker, V.H., 1983. Rate of cocoon formation and its physiological correlates in a ceratophryd frog. Physiol. Zool. 56, 430–435.

Meyer, K., 1947. The chemistry of the mesodermal ground substance. Harvey Lectures Ser. 51, 88.

Natochin, Yu, V., 1960. Morphodynamic equivalents of the reaction of the kidney of vertebrates to dehydration. In: Evolution of Physiologic functions, Proceedings of the 2nd Scientific Conference Dedicated to the Memory of Academecian L.A. Orbeli, pp. 173.

Overton, J., 1969. A fibrillar intercellular material between reaggregating embryonic chick cells. J. Cell Biol. 40, 136–143.

Patankar, V.B., Patil, V.Y., 1983. Mucopolysaccharide histochemistry of chiropteran glomerulus. Z. Mikrosk. Anat. Forsch. Leipzig. 97, 713–718.

Pearse, A.G.E., 1953. Histochemstry, Theoretical and Applied. Churchill, London.

Pessac, B., Defendi, V., 1972. Cell aggregation: role of acid mucopolysaccharide. Science 175, 898–900.

Ratner, M.Y., Tomilina, N.A., 1966. On the effect of antidiuretic hormone on mucopolysaccharides in interstitial substance of skin. Dokl. Akad. Nauk. SSSR 170, 1226–1228.

Reno, H.W., Gehlbach, F.R., Turner, R.A., 1972. Skin and aestivational cocoon of the aquatic amphibian, siren intermedia le conte, Copeia, 625–631.

Rogers, H.J., 1961. The structure and function of hyaluronate. Symp. Biochem. Soc. 20, 51–78.

Ruibal, R, Hillman, S.S., 1981. Cocoon structure and function in the burrowing hylid frog, *Pternohyla fldiens*. J. Herpetol. 15, 403–408.

Shoemaker, V.H., Hillman, S.S., Hillyard, S.D., et al., 1992. Exchange of water, ions and respiratory gases in terrestrial amphibians. In: Feder, M.E., Burggren, W.W. (Eds.), Environmental Physiology of the Amphibia, University of Chicago Press, Chicago pp. 125–150.

Shylaja, R., Alexander, K.M., 1974. Studies on the physiology of excretion in the fresh water prosobranch, *Pila virens*: part I. Pattern of excretion in the normal and post aestivating *P. virens*. Indian J. Exp. Biol. 13, 363–365.

Slavkin, H.C., Bringas, P., Cameron, J., LeBaron, R., Bavetta, L.A., 1969. Epithelial and mesenchymal cell interactions with extracellular matrix material in vitro. J. Embryol. Exp. Morphol. 22, 395–405.

Van Beurden, E., 1982. Desert adaptations of Cyclorana platycephalus: a holistic approach to desert adaptation in frogs. In: Barker, W.R., Greenslade, P.J.M. (Eds.), Evolution of the Flora and Fauna of Arid Australia, Peacock Publications, South Australia pp. 235–240.

Van Beurden, E., 1984. Survival strategies of the Australian

water –holding frog, Cyclorana platycephalus. In: Cogger, H.G., Cameron, E.E. (Eds.), Arid Australia, Australian Museum, Sydney pp. 223–224.

Wiederhielm, C.A., Fox, J.R., Lee, D.R., 1976. Ground substance mucopolysaccharides and plasma proteins: their role in capillary water balance. Am. J. Physiol. 230, 1121–1125.

Withers, P.C., 1995. Cocoon formation and structure in the aestivating Australian desert frogs *Neobatrachus* and *Cyclorana*. Aust. J. Zool. 43, 429–441.

Withers, P.C., 1998. Evaporative water loss and the role of cocoon formation in Australian frogs. Aust. J. Zool. 46, 405–418.

Zimny, M.L., Hollier, L., Clement, R., 1971. Ultrastructure of the proximal convoluted tubule of a hibernator correlated with renal enzymology. Comp. Biochem. Physiol. A 40, 405–414.

Zimny, M.L., 1973. Mucopolysaccharide in the glomerular basement membrane of a hibernator. Am. J. Anat. 138, 121–125.