ACTIVE SODIUM UPTAKE BY THE TOAD AND ITS RESPONSE TO THE ANTIDIURETIC HORMONE

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It has long been known that extracts of the neurohypophysis have a profound influence on the water economy of amphibians. Brunn (1), who made the initial contribution to this field, attributed the increased water content of frogs treated with extracts of the posterior lobe to an increased uptake of water through the skin. There has been considerable discussion as to the exact mechanism of the increase in water content. Ewer (2, 3) has recently demonstrated that posterior lobe extracts given to the toad produce an antidiuretic effect and cause reabsorption of water from the bladder in addition to promoting uptake through the skin. There is general agreement, however, that amphibians treated with neurohypophyseal extracts exhibit an increased water uptake. The subject is adequately discussed in Jørgensen's (4) comprehensive paper.

Regarding the mechanism of water passage through the skin, Koefoed-Johnsen and Ussing (5) have shown that the increased passage of water through the isolated skin of the toad caused by neurohypophyseal extracts is primarily dependent on increased flow rather than increased diffusion. Such an interpretation implies an increase in pore size rather than the presence of an increased area for diffusion.

Fuhrmann and Ussing (6) have demonstrated that various neurohypophyseal extracts, including the posterior pituitary of the whale, increase the potential difference across the isolated skin of the frog when applied to the inside (corium) of the skin. The particular advantage derived from using the whale pituitary is that there is a sharp anatomical division between anterior and posterior lobes. When an extract of the anterior lobe was used as a control, the above effect was not observed. It was noted that sodium influx tended to rise with a rise in the potential difference. Water uptake was also enhanced by the presence of the hormones. In these experiments the epithelial side of the skin was bathed in one-tenth frog Ringer, and the corium side in full strength Ringer.

The aim of the present paper is to try to correlate these findings on isolated

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skin with effects on the intact animal. In particular, we desired to investigate the influence of antidiuretic hormone on salt and water transport in an isotonic saline solution. No information is available for this medium.

Methods

The common toad, *Bufo bufo*, was employed as the experimental animal. Males and females, weighing between 20 and 35 gm., were used. The experiments were conducted during the late spring and summer. Animals were kept in tap water in individual beakers in a cool, basement room. They were not given food.

To measure sodium uptake the toads were anesthetized in a 5 per cent urethan solution, suspended in baths of 140 ml. of frog Ringer solution, containing radioactive sodium and ½ per cent urethan. Air was bubbled into the solution through glass wool, forming an atmosphere of fine bubbles. This kept the bathing solution well stirred, and, it is believed, allowed the animals to respire through their skins during the bathing periods. A simple device was employed to suspend the animals in the bath: A short piece of rubber tubing was split along one side of its long axis and wrapped around the middle of each foreleg. Another, slightly larger, piece of tubing was passed over the distal end of the foreleg to cover the split tubing, thus securing the extremity. The free end of the larger tubing was fixed to a glass rod, the height of which could be adjusted. When the toad was immersed in the bath the anterior tip of the mouth was adjusted to a mark on the containing vessel. This may be done conveniently by using a 250 ml. graduate as the bath chamber. The toads were suspended at a height such that the fluid level was below the posterior limit of the oral cavity.

Sodium uptake was determined by making a count of the radioactivity of the whole animal after definite periods in the bath and calculating sodium influx on the basis of animal *vs.* bath activity. In "counting" the whole animal advantage was taken of the strong gamma radiation of Na^{24}. A steel box was built in order to achieve a standard geometrical pattern. Its dimensions were as follows: height, 21 cm.; width, 17 × 17 cm.; thickness 2.4 cm. The box was built to house a 600 ml. beaker which fitted into a wooden recess at the bottom. The diameter of the beaker was 8 cm. The toad was placed in the bottom of the beaker, lying always in the same position and along the same axis of the box. The window of a monitor tube was centered over the toad at a height of 18 cm. above the floor of the box. The tube was a conventional end-window Geiger-Müller counter. A daily check of this counting system was made by using Sb^{125}, a gamma emitter with a half-life of 2.7 years. The Sb^{125} count did not change appreciably over a period of 2 months. The animals were counted for two successive 8 minute periods. Ordinarily the difference between duplicate counts did not exceed 5 per cent, including the statistical counting error which varied from 1 to 3 per cent.

It was also necessary to determine the Na^{24} content of solutions, using the chamber and beaker described above, for two purposes; to calculate the amount of active sodium to add to the bathing solutions, and to measure the sodium outflux of the toad. Using 50 ml. of water and a few microliters of radioactive stock solution the counts obtained were directly proportional to the amount of activity in solution. For the sodium outflux experiments a 50 ml. aliquot of the bathing solution was

counted. It was found that toads injected with a known amount of radioactive sodium gave a consistently higher count than was obtained from the same amount of activity counted in solution. A correction factor was introduced to compensate for this discrepancy. For toads of the size used this factor was 0.83; *i.e.*, toad counts were multiplied by 0.83 to make them comparable to known amounts of activity.

Radioactive sodium was obtained as weightless Na^{24} from Philips-Roxane, Amsterdam, and as $Na_2^{24}CO_3$ powder from Harwell, England. The $Na_2^{24}CO_3$ was converted to isotonic $Na^{24}Cl$ before use.

The antidiuretic hormone used was insipidin AB, a preparation of ox posterior pituitary containing 20 I.U. of the vasopressor substance per ml. Control injections were made with the same substance, made biologically inactive by autoclaving at an alkaline pH. Both preparations were made up in 0.9 per cent saline. Injections consisted of 100 μl. of control or active material. They were made subcutaneously between the nares in such a position that any break in the skin would be above the fluid level of the bathing solution.

The procedure for measuring sodium influx may now be summarized. The toad was anesthetized for 15 minutes in a 5 per cent urethan solution. He was then catheterized to remove urine, dried gently with filter paper, and weighed. The weighings had an accuracy of about 10 mg. The animal was then suspended in the bathing solution for 1 hour, removed, dried, and weighed. The animal was counted as described above. After counting the toad was injected with either the control or active hormone preparation, weighed, and immersed in the same bath for a 3 hour period. At the end of this time it was removed, weighed, and counted again. The 1 hour period served as a base line for any changes apparent after the 3 hour period. Recovery of the toads was enhanced by placing them into shallow dishes supplied with a stream of tap water.

For the outflux experiments radioactive sodium was introduced into the toad's stomach through a polyethylene catheter. Equilibrium of Na^{24} distribution was determined by counting the whole animal until successive counts became constant, usually 2 to 4 hours after intubation. The procedure was then essentially the same as in the influx experiments except that the bathing solutions contained no radioactive sodium at the start of each bathing period, and sodium outflow was measured by counting aliquots of the bathing solution after each 1 hour and 3 hour period.

To measure electrical potential a Ringer-agar bridge was inserted into the toad's dorsal lymph sac through a small skin incision just posterior to the head. The toad was immersed in the bathing solution, and a similar bridge was inserted into the bath. The two bridges were connected through saturated KCl solutions to calomel electrodes. Readings were made on a valve potentiometer (Radiometer, Copenhagen). This is the same method used by Jørgensen *et al.* (7) who give further details in their paper.

All bathing solutions contained 0.5 per cent urethan to maintain anesthesia. For sodium influx and outflux experiments the baths consisted of frog Ringer plus radioactive sodium when appropriate. For some of the studies on water balance isotonic and hypertonic sucrose were used. Isotonic sucrose was made up to contain 8.03 gm. sucrose per 100 ml. of aqueous solution. Hypertonic sucrose contained 12.00 gm. of sucrose per 100 ml. of aqueous solution.

An estimation of sodium space was made in the following way: A known amount

of radioactive Na^{24} was introduced into the stomach of a toad of known weight. When the whole-animal count became constant, about 75 μl. of lymph was withdrawn from the subcutaneous lymph space through a capillary tube. The specific activity of the lymph was measured by drying two 25 μl. samples and counting them with a conventional Geiger-Müller counter. The total activity of the sodium originally introduced was determined by counting an aliquot in the same counter. The activity of 1 μl. of lymph divided into the total activity introduced gave a measure of the sodium space. This turned out to be 29 per cent of the body weight, a figure which was then used for all calculations of sodium space. Total sodium was estimated from sodium space, using 100 microequivalents per ml. as a reasonable sodium concentration for the toad. The correctness of this assumption was supported by a few sodium determinations on toad sera.

RESULTS

Toad surface areas were calculated from the formula of Rey (8):

$$\text{Surface area} = (6.0)\ (\text{weight})^{2/3}$$

Sodium Influx.—Table I shows the results of seven experiments on sodium uptake. The animals were paired, and an experimental and control toad were carried through the procedure simultaneously, using duplicate baths. The formula used was:

$$\frac{\text{Toad C.P.M.} \times 111}{\text{Bath C.P.M./ml.}} = \mu\text{e. Na taken up.}$$

"Toad C.P.M." is the count per minute of the whole animal corrected to correspond to a solution count. "Bath C.P.M./ml." was the count per minute of 1 ml. of bathing solution based on the activity of the radioactive sodium added. The value "111" is the approximate value for the microequivalents of sodium in 1 ml. of frog Ringer.

Toads treated with insipidin showed an increased uptake of sodium over the control hour, ranging from 34 to 103 per cent. The control animals showed marked or slight decreases in sodium uptake after injection, except for toad 10 which showed a marked increase, possibly due to moulting. It should be noted that toads 5 and 6 served alternately, as experimental and control animal, each animal showing this characteristic response to the hormone. Toad 11 was given saline as a control injection to ensure that no depressant substance was present in the inactivated insipidin.

Sodium Outflux.—Table II contains data from outflux experiments. Outflux was calculated from the equation:

$$\frac{\text{Bath C.P.M.}}{\text{Toad C.P.M.}} \times 100 = \text{per cent toad sodium}$$

TABLE I

Effect of Antidiuretic Hormone on Sodium Influx

Experiment No.	Toad	Surface area	Time in bath	Treatment*	Sodium influx per cm.² per hr.	Per cent change	Weight change
		cm.²	*hrs.*		*μeq.*		*mg. per cm.² per hr.*
1	1	54.7	1	None	0.89	—	3.4
	1	54.7	3	Insipidin	1.25	+40.0	9.0
	2	50.0	1	None	0.68	—	2.6
	2	50.0	3	Control injection	0.75	+10.0	1.9
2	3	57.4	1	None	0.58	—	0.9
	3	57.4	3	Control injection	0.57	−1.7	3.7
	4	50.4	1	None	0.89	—	3.5
	4	50.4	3	Insipidin	1.64	+84.0	6.9
3	5	43.8	1	None	0.95	—	4.1
	5	43.8	3	Insipidin	1.43	+51.0	8.3
	6	58.2	1	None	1.63	—	10.4
	6	58.2	3	Control injection	1.32	−19.0	14.1
4	7	51.7	1	None	1.00	—	5.3
	7	51.7	3	Control injection	0.75	−25.0	7.6
	8	53.6	1	None	0.64	—	3.6
	8	53.6	3	Insipidin	1.27	+99.0	6.4
5	9	49.4	1	None	1.20	—	7.5
	9	49.4	3	Insipidin	1.61	+34.7	12.4
	10	55.7	1	None	1.51	—	5.5
	10	55.7	3	Control injection	2.02	+33.7	10.4
6	11	51.7	1	None	1.03	—	5.3
	11	51.7	3	Control injection	1.00	−3.2	7.1
	12	52.3	1	None	1.03	—	7.1
	12	52.3	3	Insipidin	2.09	+103.0	10.5
7	5	43.7	1	None	2.12	—	3.2
	5	43.7	3	Control injection	1.46	−31.0	7.8
	6	50.5	1	None	1.43	—	5.6
	6	50.5	3	Insipidin	3.40	+138.0	9.1

* Insipidin was injected subcutaneously, 100 μl. per experimental animal. The control injection consisted of 100 μl. of inactivated insipidin except in the case of toad 11 which received 100 μl. of 0.9 per cent saline.

In this expression "Bath C.P.M." was determined after each bathing period by counting 50 ml. of the bathing solution. "Toad C.P.M." was corrected, as described in the previous section, to correspond to solution counts. It represents the count of the whole animal at the start of the experiment. Total toad sodium was estimated as described earlier, and the absolute amount of sodium leaving the toad was then calculated from total sodium and per cent found in the bath. In four cases it was not possible to measure sodium outflux because the count of the bathing solution did not exceed background. In the 1 hour period for animal 8 there was an appreciable outflux of sodium. But

TABLE II

Effect of Antidiuretic Hormone on Sodium Outflux

Experiment No.	Toad	Surface area	Time in bath	Treatment	Sodium outflux per cm.² per hour	Total outflux as per cent of total toad sodium	Weight increase
		cm.²	*hrs.*		*μeq.*		*mg. per cm.² per hr.*
1	7	52.2	1	None	0.13	0.90	7.50
	7	52.2	3	Insipidin	0.02	0.15	4.40
	8*	53.6	1	None	0.56	4.60	—
	8	53.6	3	Control injection	Not detectable	—	4.30
2	1	50.0	1	None	Not detectable	—	—
	1	50.0	3	Insipidin	Not detectable	—	8.60
	3	52.8	1	None	Not detectable	—	—
	3	52.8	3	Control injection	0.02	0.10	6.60

* This animal released its moulted skin into the bath during the 1st hour.

this value is probably too high since the bath was contaminated by the toad's moulted skin. In the two other animals for which calculation was possible the sodium outflux does not appear to be very large for either experimental or control animal.

Water Balance Studies.—In the last column of Tables I and II weight changes are listed for each animal for each bathing period. In almost all cases the hourly weight increase is greater during the 3 hour period. This increase was obviously due to water uptake and experiments were carried out to see what influence different media might have on this phenomenon. Data in Table III show comparative results for different bathing solutions. In Experiment 2 a skin puncture with an injection needle was made in one animal, and nothing was done to the control. The increased water uptake of the longer period was still noted for both animals. In isotonic sucrose there appears to be a slight over-all loss of water. In hypertonic sucrose there is water loss which becomes greater during the longer period.

TABLE III

Effect of Different Bathing Solutions on Water Balance

Experiment No.	Toad	Surface area	Bathing solution	Time in bath	Treatment*	Water uptake
		cm.²		*hrs.*		*mg. per hr. per cm.²*
1	5	43.7	Frog Ringer	1	None	+3.2
	5	43.7		3	Inactive insipidin	+7.8
	6	50.5	Frog Ringer	1	None	+5.5
	6	50.5		3	Insipidin	+9.1
2	11	49.2	Frog Ringer	1	None	+0.4
	11	49.2		3	Skin puncture	+2.0
	12	47.8	Frog Ringer	1	None	+2.9
	12	47.8		3	None	+3.4
3	13	48.3	Isotonic sucrose	1	None	−1.0
	13	48.3		3	Inactive insipidin	−1.5
	14	54.6	Isotonic sucrose	1	None	+1.3
	14	54.6		3	Insipidin	−0.7
4	1	49.8	Hypertonic sucrose	1	None	−1.6
	1	49.8		3	Inactive insipidin	−7.4
	2	48.0	Hypertonic sucrose	1	None	−4.2
	2	48.0		3	Insipidin	−6.5

* Experimental and control injections as in Table I.

TABLE IV

Measurements of Electrical Potential across the Skin

Experiment No.	Toad	Bathing solution	Treatment	Potential*
				mv.
1	15	Frog Ringer	After 30 min. in bath	+22.8
			Needle puncture of skin	+23.4
			Inactive insipidin	+27.5
			Insipidin	+31.0
2	16	Frog Ringer	After 60 min. in bath	+26.7
			Needle puncture of skin	+31.2
			"Handling" of the animal	+40.5
		Isotonic sucrose	15 min. after changing bath	−22.3

* Sign refers to electrode inside dorsal lymph sac.

Potential Measurements.—Table IV shows the result of skin potential measurements on two toads. In the first experiment an attempt was made to see whether injections of active or inactive insipidin altered the potential. It will be seen from Experiment 2 that any changes observed were not significant due to the large change which occurred from mere handling of the animal. It should also be noted that the electrode inside the dorsal lymph sac of toad 16 became negative shortly after changing the bath solution to isotonic sucrose.

DISCUSSION

The method employed in this work appears to have very definite advantages with regard to the study of sodium uptake by the whole animal. It would be very difficult indeed to detect and measure sodium uptake from frog Ringer by chemical analysis of the bathing solution or of the animal. It would likewise be impractical to attempt to study the uptake of sodium by noting changes in the radioactivity of the bathing solution in which such changes are of such a small order of magnitude.

The values obtained for sodium transport using the whole animal are of the same order of magnitude as those found for the isolated skin by Ussing (9). It thus appears that the intact animal may be used to complement work done on isolated skin. In this connection the striking increase in sodium transport effected by injections of insipidin into the intact toad parallels the finding of Fuhrmann and Ussing (6) using isolated frog skin.

That the hormone really increases active transport and not merely exchange of sodium ions is evident from the very low values obtained in the sodium outflux studies. For the four cases in which there was a measurable increment of Na^{24} in the bathing solution the average value is 0.19 μeq. Na per cm.^{2} per hour. This may be considered an upper, limiting value since it does not include cases in which outflux was too low to measure. This average value is only 15 per cent of the lowest value for Na uptake after hormone injection, 1.25 μeq. Na per cm.^{2} per hour.

Water uptake occurred in both experimental and control animals during the sodium transport experiments. From the data in Table I it is seen that the increase in the hourly rate of water uptake which occurs during the 3 hour period is greater for the animals treated with the active hormone preparation. There is an average weight increase of 2.9 mg. per cm.^{2} per hour for control toads, and an increase of 3.9 mg. per cm.^{2} per hour for experimental toads. That the water uptake is related to sodium transport appears evident from Table III. In isotonic sucrose, no such water uptake was observed. Hypertonic sucrose was used to see whether an independent, positive effect of insipidin on water uptake would be seen in a hypertonic medium. This did not appear to be the case.

But water uptake does not closely follow sodium uptake. In the control animals the rate of sodium uptake tended to fall during the 3 hour period, but water uptake increased.

To correlate these findings, it is suggested that the primary influence of the antidiuretic hormone under the experimental conditions is to cause an increased uptake of sodium. The fact that the increase in water uptake is larger in the toads treated with hormone is probably due to a "drag" effect of the increased sodium influx. To support this is the observation that insipidin does not increase water uptake in isotonic sucrose. Still unexplained is the increase in water uptake by control animals. The only suggestion we can offer at present is that something in frog Ringer solution progressively affects toad skin permeability without enhancing sodium transport.

Potentiometric measurements of the skin potential using isolated frog skin have been made by Ussing (9), who has shown that the potential is due primarily to sodium transport. The values obtained in the intact animal are of the same sign and order of magnitude. However, it cannot be concluded that sodium transport is the main source of the potential observed in the intact animal without making simultaneous measurements on influx and outflux of both sodium and chloride. It was hoped that an increased skin potential might be observed after insipidin injection. That this was not observed may be due to an increase of chloride permeability accompanying enhanced sodium transport. An inverse relationship has been found to exist between chloride permeability and potential (10).

It should be pointed out that all data mentioned in the discussion relating to sodium transport in isolated frog skin have been verified for the isolated toad skin by Andersen and Ussing (11).

SUMMARY

A method has been described in which sodium uptake may be studied in the intact, anesthetized toad. Sodium uptake is determined by "counting" the whole animal in a special chamber after suspending it in a frog Ringer bath containing radioactive Na^{24}. The effects of subcutaneous injection of the neurohypophyseal antidiuretic factor were studied with these results:

1. There was a pronounced increase in sodium influx following treatment with the hormone.
2. Sodium outflux was small in both experimental and control animals.
3. There was an increase in water uptake in both experimental and control animals after 1 hour in the bathing solution. This increase was greater in the experimental toads in which it is believed to be related, at least in part, to sodium transport.
4. Potentiometric measurements were made on the skin membrane potential of the whole animal while suspended in bathing solutions. These results

were in essential agreement with those found for isolated frog skin. However, there was no apparent influence of the antidiuretic factor on the skin potential.

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