RELATION OF METABOLISM OF FROG SKIN TO CELLULAR INTEGRITY AND ELECTROLYTE TRANSFER*

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Isolated frog skin maintains for many hours an efficient salt pump which accumulates sodium chloride at the inside of the skin (1). There is definite evidence that active, specific sodium transport is at work (2). Such active transport obviously requires an energy supply. A close relationship between oxidative metabolism and the transport mechanism has been shown by Huf (3, 4).

We have studied the energy requirements of active sodium transport, with particular reference to the role of high energy phosphate bonds. To this purpose we have made use of a number of agents whose effects on metabolism have been characterized by other means. It appears from these studies that phosphate bond energy is necessary for sodium transport by frog skin. In the course of the experiments it has also become clear that phosphate bond energy is necessary for the maintenance of the high intracellular concentration of potassium characteristic of the skin cells themselves. However, our results indicate that the mechanisms of sodium transport by the cells and of potassium accumulation within the cells of the frog skin are independent.

Methods

The skin bag method described by Huf (3, 4) was used with only minor modifications. Each bag was formed from the skin of the hind leg of a frog. The activity of each experimental bag was compared with that of a control bag made from the skin of the other leg of the same frog. As Huf (1) has shown, and as we have confirmed, two bags from the same frog under the same experimental conditions differ in activity by less than 5 per cent. In these experiments, only "rightside-out" bags, in which the natural outside of the skin forms the outside of the bag, have been used.

Experiments were run for 18 hours in a constant temperature room at 16°C. Each experiment consisted of four experimental bags and their controls. The experimental group and the control group were each suspended in 1.5 liters of a special Ringer's solution. This fluid is referred to below as the "bath." At the start of an experiment, 4 or 5 ml. of fluid were pipetted from the bath into each bag. This fluid is referred to

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below as the "bag" Ringer's. (The amount of bag fluid depended on the size of the skin bag. In any one experiment, all bags contained the same amount of fluid.) The procedure employed ensured that bag and bath fluids had the same composition and concentrations at the start of an experiment.

All the Ringer's used in any one experiment was made up in one lot from stock solutions. It was so made up that the concentrations of ions (μeq./ml.) were approximately as follows: Na^+48, K^+0.8, Ca^{++}2, Cl^-50, HCO_3^-0.8. This Ringer's, 0.4 its "physiological" strength, has been shown by Huf (1) to give the maximum sodium gradients across the skin. Exact concentrations of sodium and potassium were determined by internal standard flame photometry. Chloride was determined potentiometrically (5). The error of these estimations is $\pm$2 per cent. pH determinations accurate to $\pm$0.02 unit were made. Volume changes in the bag fluids were determined by weighing, with a correction for fluid simply adhering to the bag. This method is accurate to about 0.1 ml.

Materials under investigation were dissolved in the bath containing the experimental bags. The pHs of experimental and control baths were then adjusted to the same level with NaOH and HCl as necessary. Inasmuch as bag fluid was pipetted directly from the bath, the experimental material was present in the same concentration in bag and in bath at the start of an experiment.

Oxygen consumption of the skins was determined by the conventional direct Warburg technique. Skins were suspended in our Ringer's solution and oxygen consumption was determined for at least 1 hour to obtain the control value. The experimental material was then added from the side arm of the Warburg vessel and the experimental value determined. Constancy of oxygen uptake by control skins during periods up to 3 hours was demonstrated in separate experiments.

The values in an experiment were determined separately for each bag and the results then treated statistically. Numerical results are expressed as arithmetic means plus or minus the standard errors of the means.

RESULTS AND DISCUSSION

Action of DNP on Sodium Transport

The role of phosphorus compounds as the energy source for numerous biological activities, including transfer mechanisms (6, 7), is well defined. We have studied the possible role of high energy phosphate bonds in meeting the energy requirements of active sodium transfer by the frog skin. To this end we have employed 2,4-dinitrophenol (DNP), whose action of preventing the generation of phosphate bonds has been extensively studied (8, 9).

Fig. 1 shows the effects of DNP on the net transfer of sodium or chloride by the skin, and on the ability of the skin to maintain across itself a gradient of these ions. Per cent inhibition is calculated as equal to [(control—experimental)/control] $\times$ 100. Concentrations of DNP as low as 10^{-5} molar may be seen to inhibit the transport of salt by the skin. Determinations of oxygen uptake show increased consumption by skins treated with DNP. This is true even at the highest concentrations tested, at which sodium transport is more

than 90 per cent inhibited. Thus, at $2 \cdot 10^{-4}$ molar DNP, the Q_{O_2} (wet weight) is increased about 75 per cent, from 0.22 to 0.38.

The complete data for a representative experiment with DNP are given in Table I. The transport of sodium and chloride may be seen to follow one another closely, whether expressed as net transfer ("corrected" for osmotic water flow, according to Huf (1)) or as gradient established. The degree of inhibition produced by DNP may also be seen to be almost exactly the same, whether expressed in terms of gradient or of net transfer of sodium or chloride. These statements hold throughout the range of DNP concentrations tested. For this

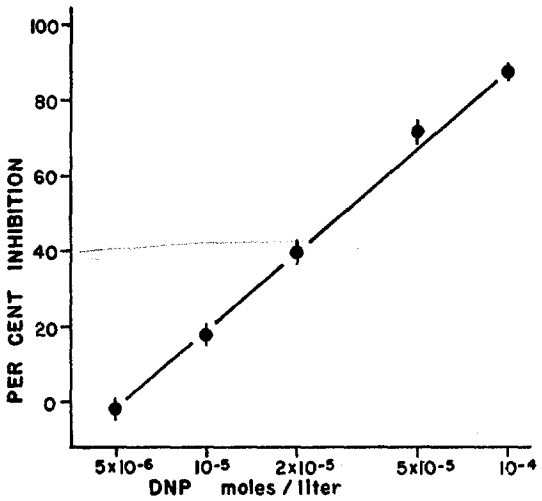

FIG. 1. Inhibition of ion transport by DNP. Points represent means, vertical lines through the points the standard errors of the means. The ordinate may represent per cent inhibition of either gradient or net transport of sodium or chloride (see text).

reason the ordinate in Fig. 1, "per cent inhibition" may refer equally well to the gradient or to the net transfer of sodium or chloride.

The fluid in the bag normally becomes more alkaline during an experiment, as H^+ is transferred from inside out. The ability of DNP-treated skins to make the bag fluid more alkaline is decreased. At levels approaching complete inhibition of sodium transport, the fluid in the bag actually becomes more acid during an experiment. Due to inhibition of the ability of the skin to transfer H^+ out, or to increase in production to levels above the transfer maximum for H^+, or to a combination of these factors, acid metabolites (probably mostly CO_2, according to Huf (1)), collect in the bag fluid.

Potassium is transported from bag to bath in control experiments. The significance of the apparent reversal of potassium transfer seen in DNP-inhibited skins will be discussed in the next section.

These results would seem to implicate phosphate bonds in meeting the energy requirements of active salt transport by frog skin. Huf (10) has reported that "the salt pump . . . does not make use of the energy . . . of added ATP." In his experiments he found no increase in sodium transport when adenosinetriphosphate (ATP) was added to the bag fluid. In our opinion, these experiments do not support the conclusion quoted, on two counts. First, there is reason to believe that organic phosphorus compounds cannot penetrate cell membranes (11). ATP itself has been shown to be hydrolyzed at the yeast cell membrane without entering the cell (12). Second, an energy source, such as ATP, even were it to enter the cell, would be expected to increase sodium transport only if the existing rate of transfer were limited by the available energy supply. There is no evidence that under ordinary circumstances this is true in the frog skin. It is at least equally likely that the transfer mechanism itself is the

TABLE I

Data for a Representative DNP Experiment

	Bag fluid					Gradient		Net transfer	
	Na	Cl	K	pH	Volume	Na	Cl	Na	Cl
	μeq./ml.				*ml.*	*μeq./ml.*		*μeq.*	
Start	47.5	45.5	0.90	7.30	5.0	—	—	—	—
DNP, $2 \cdot 10^{-5}$	69.2	67.1	1.28	7.65	5.1	21.6	21.7	109	108
Control	88.0	86.0	0.59	7.80	5.4	40.5	40.5	203	203
Inhibition, *per cent*	—	—	—	—	—	45.6	46.4	46.3	46.5

rate-limiting factor. One would expect an added energy source to be necessarily effective only when energy production by the skin itself is impaired; *e.g.*, in a metabolically poisoned skin.

The validity of using DNP as a means for the study of the role of phosphate bond energy in transfer processes has been well documented (6, 7, 13). Admittedly, on the basis of our results alone one cannot assign a definite role to high energy phosphate bonds in the transport mechanism for sodium in frog skin. We believe that our experiments justify the conclusion that phosphate bond energy is involved in meeting the energy requirements of sodium transport by frog skin. The role of ATP specifically we believe to be undetermined as yet.

Metabolism and Skin Potassium

In the previous section an apparent reversal of potassium transport was noted in skins treated with DNP. DNP-treated skins accumulate potassium in the bag, whereas control skins accumulate potassium in the bath. This action of DNP may be accounted for in two ways. (1) The direction of potas-

sium transport by frog skin may be reversed by DNP. (2) The potassium which accumulated in the skin bag may have been lost from the skin itself. These alternatives were tested by experiments in which the potassium content of the skin, as well as of the Ringer's, was determined. The plan of the experiments was otherwise exactly as described above. At the end of an experiment, the skins were dissolved in nitric acid and analyzed for potassium.

In Table II data are given for an experiment with $2 \cdot 10^{-4}$ molar DNP. This concentration inhibits essentially all sodium transport. It is apparent that experimental skins have lost significant amounts of potassium. On a unit weight basis, experimental skins contain only 61 per cent of control amounts. The average total potassium loss by an experimental skin is 9.1 μeq. The average total potassium accumulation inside a skin bag is 8.6 μeq. Thus the potassium loss by the skin, measured directly, is large enough to account for all the gain in the bag fluid.

TABLE II

Effect of DNP on Ions in Skin

	No. of skins	Bag fluid			Skin K	Weight
		Na	K	Volume		
		μeq./ml.		*ml.*	*μeq./mg. wet weight*	*mg.*
DNP, $2 \cdot 10^{-4}$	8	43.0	2.98	4.0	0.022 ± 0.001	653
Control	8	82.9	0.28	4.3	0.036 ± 0.002	621
Start	8	42.1	0.82	4.0	0.035 ± 0.002	677

The control skins, in the course of an experiment, are exposed to physiologically hypotonic Ringer's for 18 hours. It was possible that this treatment alone might cause potassium loss. To test this, skin from the abdomen or back of frogs used as controls was analyzed directly after removal from the frog. The potassium content of these skins (labeled "start" in Table II) is seen to be the same as that of skin from control bags. The experimental procedure alone, then, does not alter the potassium content of frog skin.

Further studies with a variety of metabolic inhibitors are summarized in Table III. A clear qualitative and fairly close quantitative correlation between inhibition of oxidative metabolism and loss of potassium from the skin may be seen. (Data for DNP, which inhibits "useful" metabolism, are included in Table III, although DNP actually increases oxygen consumption.) The data presented also show that inhibition of oxidative metabolism results in inhibition of sodium transport by the skin. It thus appears that oxidative metabolism is necessary both for ion transport by the skin and for the maintenance of normal potassium concentrations within it.

Hoagland and Rubin (14) have shown that the potassium in frog skin is concentrated in the epidermis; the corium contains little if any. Using data from Table II, the minimum potassium concentration in epidermal cells can be calculated to be 35 μeq./ml. The concentration of potassium in our Ringer's is about 0.8 μeq./ml. A considerable tendency for potassium to be lost from the

TABLE III

Effect of Metabolic Inhibitors on Skin Potassium

Inhibitor	Inhibition		Skin K lost, per cent control
	O_2 uptake	Na gradient	
moles/l.	*per cent*	*per cent*	
DNP, $2 \cdot 10^{-5}$	—	45	20
DNP, $2 \cdot 10^{-4}$	—	98	39
Cyanide, 10^{-3}	91	100	30
Arsenite, 10^{-3}	83	94	33
Bromacetate, $2 \cdot 10^{-3}$	56	91	26
Caffeine, $5 \cdot 10^{-2}$	42	100	14
Theophylline, $5 \cdot 10^{-2}$	38	88	13

TABLE IV

Action of Xanthines on Frog Skin

Xanthine	O_2 uptake, per cent inhibition	Na transfer, per cent inhibition	Skin K lost, per cent control
moles/l.			
Caffeine			
10^{-2}	0	44	0
$5 \cdot 10^{-2}$	42	100	14
Theophylline			
10^{-2}	0	34	0
$5 \cdot 10^{-2}$	38	88	13
Cyanate			
$2 \cdot 10^{-3}$	0	31	0
10^{-2}	41	96	11

cells by passive diffusion is thus present. Maintenance of normal intracellular potassium must therefore require the expenditure of energy to oppose this tendency. It is then reasonable that we have found that inhibition of energy production by the cell results in potassium loss. Since DNP causes potassium to leak from the skin, phosphate bond energy is presumably involved in the maintenance of intracellular potassium at normal levels. Our data give no hint as to how this energy is utilized to maintain cellular potassium levels. This point is discussed more fully below.

Action of Xanthines

Xanthines and related compounds influence ion transport mechanisms both *in vivo* (15) and in kidney slices (13). We have investigated the action of these compounds on ion transport by frog skin; the results are summarized in Table IV. These compounds are seen to inhibit sodium transport and oxygen uptake and to cause loss of potassium from the skin. However, the concentrations necessary to accomplish these actions differ. At the concentrations necessary to inhibit sodium transport about 50 per cent, none of these drugs influences oxygen uptake or skin potassium. With a fivefold increase in concentration, oxygen uptake and potassium levels in the skin are depressed.

The inclusion of cyanate with the xanthines warrants brief comment. Dicker (15) has shown that cyanate has a renal diuretic action similar to that of the true xanthines. Cyanate also produces all the "caffeine effects" studied by Keilin (16). It is therefore of interest that cyanate acts in a manner entirely analogous with the xanthines in regard to the ionic activities of the frog skin. The possibility is suggested that the C≡N grouping is the pharmacologically active part of the xanthine molecule.

Ussing (2) has pointed out certain aspects of the sodium transport mechanism in frog skin, as follows:

> "(It is) rather likely that it is the basal cell membrane of the epithelial cells which is responsible for the active Na transport. As we have seen already many cells like muscle fibers and nerve fibers must extrude Na through their surface. As far as Na transport is concerned the difference between these cell types and the epithelial cells is that the latter are only extruding Na through the cell membrane turning inward whereas the others do this throughout their entire surface."

It would seem a reasonable hypothesis that this sodium extrusion could at the same time account for sodium transport by the cell and the maintenance of potassium within it. In this case, however, one would expect the xanthines to act on sodium transport and potassium levels in a parallel manner. From the results here presented, it appears that the xanthines act specifically on the transfer mechanism for sodium in the frog skin. On the contrary, their action in causing potassium loss from the skin seems to be non-specific and associated with inhibition of metabolism. From our results, then, it is likely that the same mechanism is not active both in sodium transport and in maintaining intracellular potassium. Evidence for the existence of independent carrier systems for sodium and potassium in the erythrocyte membrane has been presented recently by Solomon (17).

GENERAL DISCUSSION

The substances whose action on the frog skin we have described may be divided into two general classes. The first comprises those materials whose mode of action is the inhibition of energy production. All poisons of aerobic

oxidative metabolism fall into this class. By virtue of its action in inhibiting phosphate bond formation, DNP also belongs in this class. In each case, inhibition of sodium transport and loss of skin potassium are proportional to the depression of effective metabolism. Into the second class fall the xanthines and related substances, which act on sodium transport in a more specific manner. Materials in this class inhibit sodium transport at concentrations well below those necessary to depress metabolism. Although it seems likely that the xanthines act on the specific transfer mechanism for sodium in frog skin, the means by which they act cannot as yet be even suggested.

We have demonstrated the importance of phosphate bond energy both in sodium transport by skin cells and for potassium maintenance within them. These facts are consistent with the known role of this kind of energy in other transfer processes, notably in the mammalian kidney (6, 7). Even in skins whose ability to transport sodium has been entirely inhibited by DNP, and in which maximal potassium loss has been produced, oxidative metabolism continues at a rate much greater than normal. This argues strongly that more than simple cell viability is involved in the inhibition of these ion transfers by DNP. The way in which phosphate bond energy is expended in these transfers is wholly unknown. In a general way, it may be assumed that the energy is involved in the formation or "activation" of the ion carrier molecule. The components of the transfer mechanism are as yet uncharacterized, and the specific role of phosphate bonds therefore cannot be defined further.

In a review of the problems of ion distribution in living organisms, Krogh (18) has stated succinctly the core of our present work:

> "It is contended that where large differences in concentration of single ions are maintained between organisms and the surrounding water or between cells and the surrounding extracellular fluid, one has to do not with a true equilibrium, but with a steady state maintained against passive diffusion and requiring the expenditure of energy."

The frog skin maintains at one and the same time large differences of concentration of single ions both between the organisms and the surrounding water and between its own cells and their surrounding extracellular fluid. The maintenance of sodium within the frog and potassium within the cells of the skin, we have shown, requires the expenditure of phosphate bond energy to oppose the forces of passive diffusion.

SUMMARY

1. The transport of sodium by frog skin and the maintenance of normal potassium levels within the cells of the skin are both dependent on metabolism and on a supply of phosphate bond energy.

2. Except for the common requirement of phosphate bond energy, the trans-

fer mechanism for sodium and the mechanism for maintaining intracellular potassium appear to be independent.

3. The xanthines specifically inhibit the sodium transfer mechanism; at higher concentrations, they depress metabolism and cause loss of potassium from the skin.

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