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A DUAL EFFECT OF SODIUM ON OXYGEN CONSUMPTION IN TOAD BLADDER

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SUMMARY

1. Q_{O_2} measured in a Warburg respirometer was found to be stimulated by the addition of external Na^+. The relationship between extra Q_{O_2} and the concentration of Na^+ displays saturation kinetics, the maximal effect occurring at 40–60 mM.

2. 2,4-Dinitrophenol stimulated Q_{O_2} and inhibited the short-circuit current (s.c.c.). The maximally effective concentration of dinitrophenol was the same for both, $5 \cdot 10^{-5}$–$10 \cdot 10^{-5}$ M.

3. The addition of Na^+ (40 mM) in the presence of dinitrophenol ($1 \cdot 10^{-4}$ M) produced a further marked stimulation of Q_{O_2}. The effects of varying the concentration of dinitrophenol on this Na^+-dependent Q_{O_2} paralleled the effects on s.c.c. and Na^+-independent Q_{O_2}.

4. The results are interpreted as showing a direct stimulation of the respiratory chain by Na^+ *per se*.

INTRODUCTION

The rate of O_2 uptake by tissues is thought to be limited by the rate of the energy conservation reactions which are coupled to the respiratory chain[9]. In the presence of an uncoupling agent, *e.g.*, 2,4-dinitrophenol, this limitation is removed and respiration proceeds at near maximal rates. It is then limited only by substrate supply[11] and the rate at which reducing equivalents can flow through the respiratory chain[9,18].

Respiration in the urinary bladder of the toad has been shown to be controlled in part by the rate of active Na^+ transport (S. HERSEY, unpublished results; refs. 4 and 5). The mechanism by which this control is exerted, however, has not been demonstrated. One current hypothesis is that respiratory control is exerted *via* ADP produced during the transport process[6,7]. According to this hypothesis, then active transport exerts its effect on the energy conservation reactions rather than on the respiratory chain itself.

If both dinitrophenol and active Na^+ transport are acting on the energy conservation reactions, one would expect a competitive interaction between them with respect to enhancing O_2 consumption. Thus, the increment in respiration produced by dinitrophenol should be greater in the absence of active transport than in its presence. If, on the other hand, active transport influences the respiratory chain directly, no such competition would be expected. The purpose of this study was to test for com-

petition between dinitrophenol and active Na^+ transport as evidence for the ADP hypothesis in the toad bladder.

MATERIALS AND METHODS

Toads (*Bufo marinus*) were obtained from Lemberger (Oshgosh, Wisc.) and kept in moist sand for periods of up to 6 weeks prior to use. In preparing the bladders, care was taken to remove excessive amounts of blood, since amphibian red blood cells consume O_2[11], and their presence might complicate interpretation of the results. To accomplish this, the toad was injected with 1 ml of a heparin solution (100 units/ml in 0.1 M MaCl) 1–2 h prior to sacrifice. The toad was then decapitated, pithed and the hind quarters were perfused with Ringer solution *via* the descending aorta. A perfusion pressure of 50–60 cm H_2O was employed, and it usually required 300–600 ml of fluid to clear the bladder adequately. The bladder was then removed quickly. In those experiments where the effect of Na^+ on O_2 consumption was to be determined, the bladders were preincubated in a Na^+-free Ringer solution. The preincubation consisted of two washes of 15 min each. This insured removal of both the extracellular and most of the intracellular ions. In the other experiments, the standard Ringer's solution was used for the preincubation. The individual half-bladders were then mounted in a chamber similar to that described by USSING AND ZERAHN[3] or cut into small pieces for use in the Warburg apparatus.

The short-circuit current (s.c.c.) was used as a measure of active Na^+ transport[1,3]. Spontaneous potential differences were monitored with calomel half cells connected to the chamber by satd. KCl bridges. s.c.c. was passed through carbon-clad copper electrodes which were also connected to the chamber by satd. KCl bridges. Both the potential differences and s.c.c. were recorded on a Grass Polygraph (Model 5, Grass Instr., Quincey, Mass.). The tissue was kept continuously short-circuited except for brief (5 sec) times in which the current was turned off to measure the potential differences.

O_2 consumption was measured in a Warburg respirometer using standard manometric techniques[12]. Following the preincubation, the bladders were cut into eight pieces and each piece placed in a separate vessel. A sufficient number of toads (usually 3–4) were used in each experiment to provide 20–30 mg dry tissue per vessel. Dry weights were determined by leaving the tissue overnight at 100°.

Solutions were prepared from reagent grade chemicals whenever possible. The standard Ringer's solution contained 117 mM Na^+, 5mM K^+, 123 mM Cl^-, 2 mM Ca^{2+} and 2 mM phosphate buffer (pH 7.2). Solutions containing reduced Na^+ were prepared by replacement with choline, on a mole for mole basis. All solutions were fortified with 3 mM pyruvate. Sodium azide and 2,4-dinitrophenol were obtained from Sigma Chemical Co.

RESULTS

Effect of sodium addition

The occurrence of active Na^+ transport could not be directly measured in the Warburg vessel. However, it was ssumed to correspond to the presence or absence of external Na^+. Since knowledge of the exact rate of active transport is not required for these experiments, there is no need to measure it directly. Supportive evidence for

the existence of active Na^+ transport in the respirometer was obtained from measurements of the extra O_2 consumption produced by the addition of external Na^+. The effect of external Na^+ on respiration measured in the Warburg apparatus is illustrated in Fig. 1. This curve relating the Na^+ induced increment in O_2 consumption to the Na^+ concentration of the bathing medium is seen to be quite similar to the relationship observed between Na^+ concentration and s.c.c. (ref. 13). The agreement between the effects of Na^+ concentration on the s.c.c. and O_2 uptake supports the assumption that in the Warburg vessel the occurrence of active Na^+ transport corresponds to the presence or absence of external Na^+.

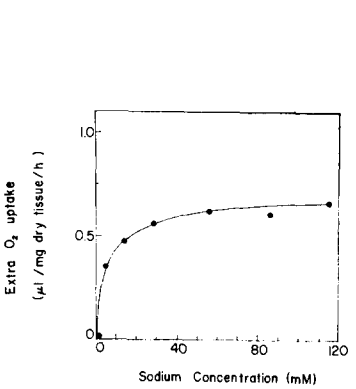


Fig. 1. The relationship between external Na^+ concentration and extra O_2 uptake. The experiment was performed in the Warburg respirometer at 24°. Each point is the average of duplicate tissue samples.

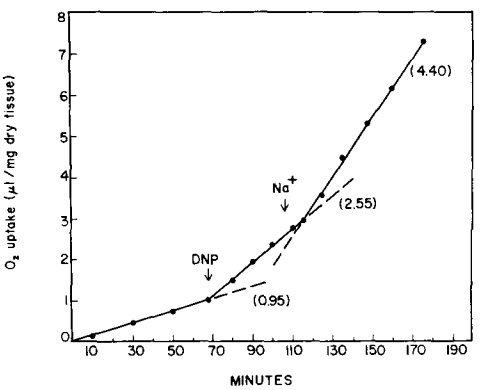


Fig. 2. Effect of sequential additions of 2,4-dinitrophenol (DNP) and Na^+ on O_2 uptake. The numbers in parentheses give the rate of O_2 uptake (μl O_2 per mg dry tissue per h). Note the linear points indicating a steady rate of O_2 consumption is reached after each addition. 21°. (See text for details.)

Interaction between dinitrophenol Na^+

Using the presence of external Na^+ as an indicator of active transport, evidence was sought for competition between dinitrophenol and active Na^+ transport. The initial experiments simply involved measuring the increment in O_2 consumption produced by dinitrophenol, either in the presence or absence of external Na^+. Although only a few experiments were performed, it was obvious that competition between dinitrophenol and Na^+ transport does not occur in the toad bladder. In fact, the increment in O_2 consumption produced by dinitrophenol was observed to be greater in the presence of Na^+ than in its absence. Since the presence of Na^+ appeared to potentiate the dinitrophenol stimulation, additional experiments were designed to test this point more directly. In these experiments a basal rate of O_2 consumption was obtained in Na^+-free medium. Dinitrophenol ($1 \cdot 10^{-4}$ M) was added, and finally a volume of normal Ringer solution, sufficient to bring the final Na^+ concentration to 40 mM, was added. A typical experiment of this type is shown in Fig. 2. The basal rate of O_2 consumption in Na^+-free Ringer solution amounted to 0.95 μl per mg dry wt. per h. The addition of dinitrophenol resulted in an increase in respiration to 2.55 μl O_2 per mg per h and the subsequent addition of Na^+ produced a further increase to 4.50 μl O_2 per mg per h. The values obtained from several similar experiments are given in Table I.

TABLE I

EFFECTS OF DINITROPHENOL AND Na^+ ON RESPIRATION

Expt. No.	*O_2 Consumption (μl O_2 per mg dry tissue per h)*		
	Control	*Dinitrophenol*	*Dinitrophenol + 40 mM Na^+*
1	0.90	3.40	4.37
2	0.90	2.65	4.20
3	0.95	2.55	4.40
4	0.90	3.40	4.45
5	1.30	2.50	3.50
6	1.35	2.70	4.45
7	1.35	2.55	4.50
8	1.22	4.24	5.93
9	1.84	4.20	5.01
Mean difference $\pm$S.E.	1.920 $\pm$ 0.220	1.420 $\pm$ 0.140	
P	0.001	0.001	

In all cases, the increments in O_2 consumption produced by dinitrophenol and by Na^+ are quite large and statistically highly significant.

Effect of varying dinitrophenol concentration

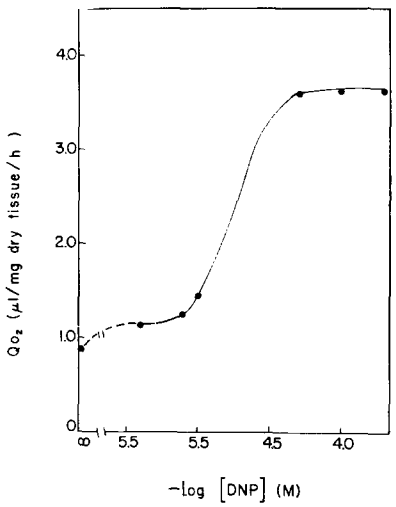
These results seem to demonstrate that Na^+ acts directly on the respiratory chain rather than *via* the energy conservation reactions. However, the possibility exists that complete uncoupling of respiration and energy conservation has not been accomplished. This seems unlikely, since the concentration of dinitrophenol employed is maximally effective, as seen in Fig. 3. The curve shows that in the absence of Na^+, a maximal respiration is produced between $5 \cdot 10^{-5}$ M and $1 \cdot 10^{-4}$ M dinitrophenol. At the concentration used in the above studies then, the extent of uncoupling is as great as can be achieved by this agent. This would seem to be sufficient evidence to exclude the possibility that Na^+ is acting *via* the energy conservation reactions.

It is also of interest that the increment in O_2 consumption produced by Na^+ is greater in the presence of dinitrophenol. The relationship between Na^+-dependent respiration and the dinitrophenol concentration is shown in Fig. 4. In these experiments, O_2 consumption was measured in Na^+-free Ringer solution containing various concentrations of dinitrophenol. A volume of normal Ringer solution sufficient to achieve a final Na^+ concentration of 40 mM was then added. The obvious similarity between Figs. 3 and 4 indicates that dinitrophenol effects the Na^+-dependent and Na^+-independent respiratory fractions in the same manner. The fact that dinitrophenol potentiates the Na^+-dependent fraction of respiration, also supports the idea of a direct effect of Na^+ on the respiratory chain. If Na^+ acted *via* the energy conservation reactions, competition between Na^+ and dinitrophenol would be expected rather than the observed potentiation.

Effect of dinitrophenol on s.c.c.

It has previously been shown that dinitrophenol inhibits active Na^+ transport in the toad bladder[2]. As seen in Fig. 5, the concentrations of dinitrophenol required to

inhibit the s.c.c. agree well with those for stimulation of respiration. Thus, under the conditions of the above experiments, very little active Na^+ transport is occurring, and it appears that the ion translocation process itself is not required for Na^+ to enhance respiration in the presence of dinitrophenol.



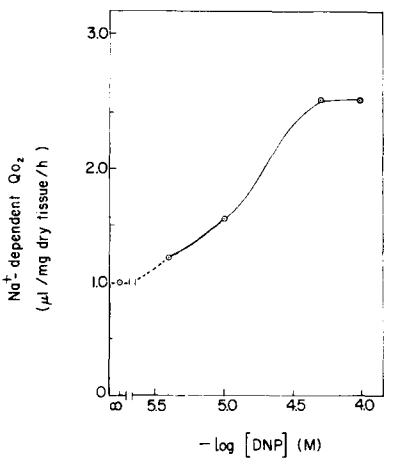


Fig. 3. Effect of 2,4-dinitrophenol (DNP) concentration on O_2 consumption in Na^+-free medium. Each point is the mean of at least four determinations. 24°.

Fig. 4. Effect of 2,4-dinitrophenol (DNP) concentration on Na^+-dependent O_2 consumption. Each point is the mean of at least four determinations. Final Na^+ concentration was 40 mM. 24°.

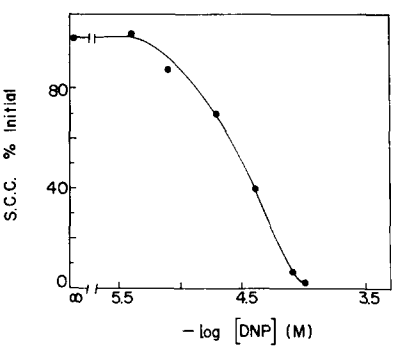


Fig. 5. Effect of 2,4-dinitrophenol (DNP) concentration on s.c.c. The initial s.c.c. was that immediately prior to adding the inhibitor. Inhibition was taken as the level attained at 30 min after adding dinitrophenol. Each point is the mean of at least five determinations. Conditions were normal Ringer's solution. 23°.

Additional characteristics of the Na^+-dependent respiration

The direct effect of Na^+ on the respiratory chain raises a number of important questions. Several lines of investigation are currently under way to further characterize this effect. The preliminary results of these investigations have provided some additional details. First, the Na^+-induced respiration in inhibited by azide, indicating that O_2 is consumed *via* a respiratory chain rather than an unknown oxygenase[14]. Sec-

ondly, there exists at least some specificity for Na^+, since the addition of K^+ in the presence of dinitrophenol does not produce a stimulation of respiration. Indeed, a slight inhibition is observed with K^+ addition. Finally, there is no inhibition of respiration by ouabain in the presence of dinitrophenol, with or without Na^+ present. This last finding is not surprising, since active Na^+ transport is not occurring under these conditions.

DISCUSSION

Stimulation of respiration by Na^+ in the toad bladder appears to be due to an effect on the rate of active Na^+ transport rather than to Na^+ *per se* (S. HERSEY, unpublished results and refs. 4 and 15). The results of the present study provide further support for this idea by showing that the effect of Na^+ concentration on Q_{O_2} parallels its effect on active transport[13]. It seems likely that this respiratory control is exerted *via* ADP produced during the transport process. Studies of O_2 uptake by isolated mitochondria have shown that ADP availability is a dominant factor in regulating respiration[9]. In addition, active ion transport is believed to involve the hydrolysis of ATP to ADP and P_i (ref. 6). This reaction is thought to be catalyzed by a monovalent cation-stimulated ATPase, which is correlated with active transport in several tissues including toad bladder[3,16,17]. The combination of ATP hydrolysis during active transport and respiratory control by ADP then provides a basis for respiratory control by active Na^+ transport in the toad bladder.

In the presence of the uncoupling agent, dinitrophenol, an additional effect of sodium ion is revealed. This appears to be a direct stimulatory effect on the respiratory chain itself. Since no active transport occurs under this condition, the stimulation of O_2 consumption seems to be due to the Na^+ *per se*. However, the possibility exists that dinitrophenol acts on the transport mechanism to dissociate the ATPase activity from ion translocation. In this case Na^+ might stimulate ADP release without any associated transport. Although this possibility cannot be ruled out, it would not appear to explain the present result. The known action of dinitrophenol is to uncouple respiration from the energy conservation reactions. In this state ADP liberated by a transport mechanism should have no effect on O_2 consumption[10]. In lieu of evidence to the contrary, the effect of Na^+ in the presence of dinitrophenol must be considered as separate from, though possibly related to, its effects in the absence of an uncoupling agent.

This dual effect of Na^+ makes it nearly impossible to observe the expected competition between dinitrophenol and Na^+ under the experimental conditions employed here. The failure to observe such competition then cannot be deemed as evidence against the hypothesis that active Na^+ transport stimulates respiration *via* ADP liberation. The validity of this mechanism must be tested, therefore, by experiments of a different type.

The stimulation of O_2 uptake by Na^+ in the presence of dinitrophenol is a quite unexpected finding. Although it demonstrates a direct effect of Na^+ on the respiratory chain, the exact nature of the effect remains unknown.

The major question involved here is the site of action of Na^+. The present results cannot distinguish whether Na^+ is acting at the substrate level or on the respiratory chain members themselves. Since the bathing media were fortified with pyruvate, a substrate effect of Na^+ would most likely be at the citric acid cycle level. This

possiblility exists as a strong one in that the rate of conversion of substrate by the dehydrogenase reactions of the citric acid cycle exert considerable limitation on respiration under uncoupled conditions[11,12]. However, because of the close relation of these reactions to the respiratory chain, it is only an arbitrary distinction to consider the dehydrogenase reactions as substrate level rather than as part of the respiratory chain.

Another and perhaps the more important, question concerning the Na^+-stimulated respiration is whether or not it is linked to active Na^+ transport. The two phenomena are obviously related by virtue of their mutual requirement of Na^+; however, any additional relationship must await further investigation.

ACKNOWLEDGMENTS

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