Effects of Dinitrophenol and Oligomycin on the Coupling between Anaerobic Metabolism and Anaerobic Sodium Transport by the Isolated Turtle Bladder

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ABSTRACT Dinitrophenol (1×10^{-5} M) has been found to inhibit anaerobic sodium transport by the isolated urinary bladder of the fresh water turtle. Concurrently, anaerobic glycolysis was stimulated markedly. However, tissue ATP levels diminished only modestly, remaining at approximately 75% of values observed under anaerobic conditions without DNP. The utilization of glucose (from endogenous glycogen) corresponded closely to that predicted from the molar quantities of lactate formed. Thus the glycolytic pathway was completed in the presence of DNP and if ATP were synthesized normally during glycolysis, synthesis should have been increased. On the other hand, the decrease in Na transport should have decreased ATP utilization. Oligomycin did not block sodium transport either aerobically or anaerobically, but ATP concentrations did decrease. When anaerobic glycolysis was blocked by iodoacetate, pyruvate did not sustain sodium transport thus suggesting that no electron acceptors were available in the system. Two explanations are entertained for the anaerobic effect of DNP: (*a*) Stimulation by DNP of plasma membrane as well as mitochondrial ATPase activity; (*b*) inhibition of a high energy intermediate derived from glycolytic ATP or from glycolysis *per se*. The arguments relevant to each possibility are presented in the text. Although definitive resolution is not possible, we believe that the data favor the hypothesis that there was a high energy intermediate in the anaerobic system and that this intermediate, rather than ATP, served as the immediate source of energy for the sodium pump.

The fresh water turtle is uniquely equipped to tolerate prolonged oxygen deprivation (1) and its urinary bladder, which shares this biological attribute, is capable of sustained anaerobic sodium transport *in vitro* (2, 3). Several observations suggest that this anaerobic transport is closely coupled to

anaerobic glycolysis. First, inhibition of forward electron transport by KCN (2) or antimycin (4) does not inhibit transbladder sodium transport markedly; whereas glycolytic inhibitors do inhibit markedly (4). Second, anaerobic glycolysis has been found to vary in a stoichiometric fashion with anaerobic sodium transport (3). Third, tissue ATP concentrations are sustained at approximately the same level during anaerobic as during aerobic sodium transport (3).

In view of these data suggesting that anaerobic metabolism serves as the energy-yielding process for anaerobic sodium transport, 2,4-dinitrophenol (DNP) would not be expected to influence anaerobic transport in a major way. DNP is a classic uncoupler of oxidative phosphorylation, and although the precise molecular basis of its action is uncertain, there is increasing evidence to indicate that it affects the hydrolysis of a nonphosphorylated high energy intermediate interposed between the mitochondrial respiratory chain and ATP (5–7). In an oxidative system, the hydrolysis of such an intermediate would account for an energy leak which would effectively "uncouple" oxidation from phosphorylation. However, high energy intermediates have not, to our knowledge, been described or implicated in the energy transfer associated with glycolysis; and in the nonnucleated mammalian red blood cell, a structure in which energy for electrolyte transport also is coupled to anaerobic metabolism, DNP does not appear to inhibit transport (8).

Despite these *a priori* considerations, DNP has been found to evoke a profound change in both metabolism and transport of the turtle bladder in an anaerobic environment. The present paper describes this phenomenon. In addition, the effects of oligomycin, an antibiotic that inhibits mitochondria-linked energy transfer, are described. The latter studies were performed in an effort to clarify the striking alterations in the biology of the bladder induced by DNP.

METHODS

The tissues used for study were obtained from the fresh water turtle *Pseudemys scripta elegans*. The urinary bladders were removed and studied in vitro under one of two conditions: (*a*) Hemibladders were suspended as a diaphragm between the two halves of a standard lucite chamber with an exposed area of 7 cm^2; (*b*) segments of bladders approximately 1 cm^2 in area were incubated in Ringer's solutions in Erlenmeyer flasks. The composition of the Ringer solution used in all experiments was as follows (all values are expressed in mM): Na^+ 114, K^+ 2.4, Ca^{++} 1.4, Mg^{++} 2.0, Cl^- 120, and $PO_4^=$ 2.4. No glucose was added to the solutions and the pH was 7.5 ± 1.

The bladders in the plastic chambers were used for the measurement of sodium transport and lactate production. The membranes were maintained in a short-circuited state except for the brief interval that was required to measure the transmembrane potential every 10 to 15 min. During the initial phase of all experiments, the Ringer solutions were gassed either with room air or with 100% oxygen until

steady-state conditions were achieved. This took from 30 min to 2 hr. Thereafter, control measurements were made in oxygenated Ringer's, in most instances, before either an inhibitor was added or anaerobic conditions were initiated. In the anaerobic studies, the gas phase was changed to 100% nitrogen and a 20 min period was allowed for deoxygenation to occur. Anaerobic measurements then were performed. Finally, inhibitors were added and additional measurements were obtained. Individual periods of observation were generally 30 min in duration and 3 or more periods were obtained under aerobic conditions and 3 or more periods were obtained under anaerobic conditions. All inhibitors were added to the serosal solution and an interval of 15 to 20 min was allowed for distribution of the drug before measurements were resumed. The concentrations of the specific inhibitors employed are described under Results.

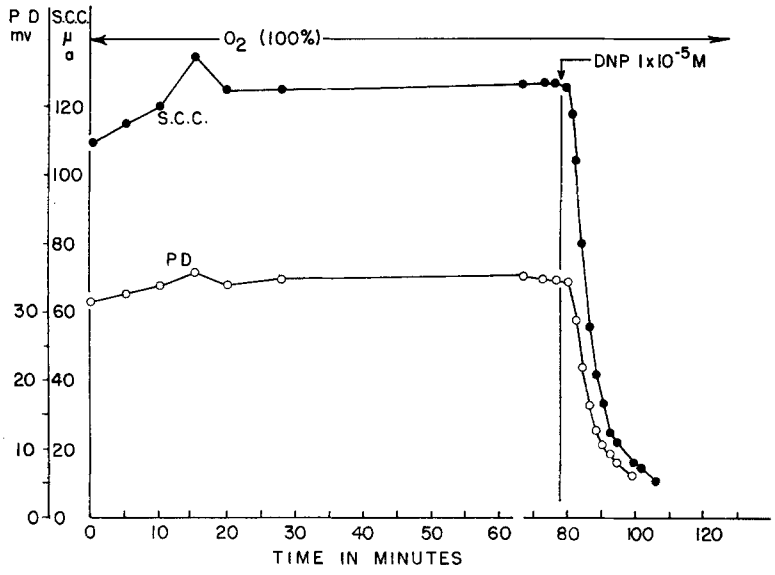
Sodium influx was measured using Na^{22} and sodium efflux using Na^{24}. The net flux was assumed to represent the difference between influx and efflux and the techniques employed have been described in detail previously (2). Both mucosal and serosal Ringer's solutions were sampled at 30 to 60 min intervals for the measurement of lactate formation.

For the studies involving the bladder segments, each bladder was cut into 8 to 10 pieces of approximately equal size and these were divided at random into 2 groups of 4 to 5 segments, each having a wet weight of about 120 mg. Each group of segments was immersed in 10 ml of Ringer's solution in an Erlenmeyer flask and the flasks were agitated gently in a Dubnoff metabolic shaker at room temperature (*ca.* 22° C). The Ringer solutions were gassed either with room air or 100% nitrogen. The inhibitor under investigation was added to one flask and the opposite flask in each set was used as a control. Incubations were continued for 1 to 2 hr. In most experiments, the lactate content of the Ringer solutions was determined, and at the end of the incubations the tissues were removed and the ATP and/or glycogen concentration measured. Similar measurements were made on control (i.e. nonincubated) bladder tissue from the same animals.

Lactate was determined using an enzymatic (LDH) method involving the conversion of DPN to DPNH (9). ATP concentrations were measured on tissue extracts using the luciferin-luciferase technique (3, 10). Glycogen was determined according to a method described previously (3). The nitrogen gas used in these experiments was passed through an oxygen trap (11) before it entered the Ringer solution. Plastic rather than rubber tubing was used to avoid O_2 contamination from the atmosphere. Attempts to measure O_2 in the deoxygenated Ringer solutions have been unsuccessful (2) and the striking changes in the biologic properties of the membrane that take place after changing the gas phase from O_2 support the view that the system was operationally anaerobic (2–4).

RESULTS

The effects of dinitrophenol on the short-circuit current (S.C.C.) and the transbladder potential difference (PD) of the isolated turtle bladder are shown in Fig. 1. Fig. 1A depicts the effects in an aerobic environment. Subsequent to the addition of DNP to the serosal solution in a final concentration of



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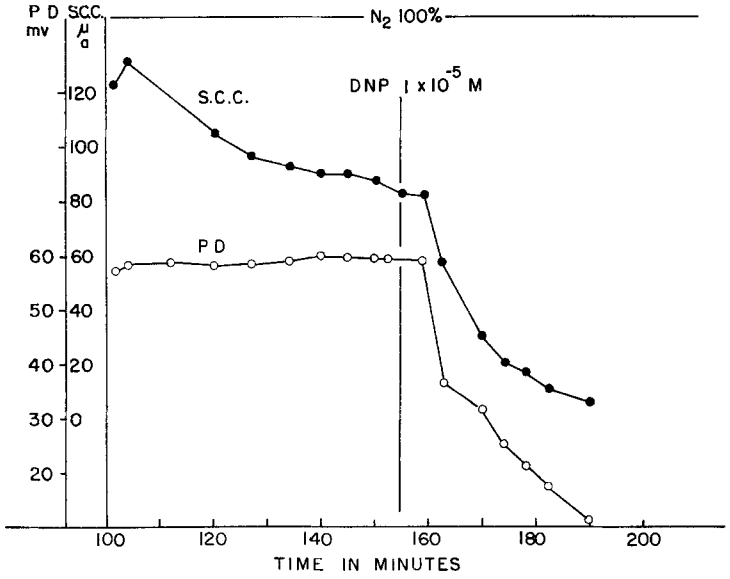

B

FIGURE 1. These figures depict the effects of the addition of DNP (1×10^{-5} M) to the serosal solution. Fig. 1A shows a representative study done in an oxygenated environment; Fig. 1B depicts a study performed under anaerobic conditions. This also was representative except for the fact that PD usually diminishes in N_2 (2). During the first portion of experiment 1B (not shown in the graph) the membrane was equilibrated in oxygenated Ringer's and 20 min were permitted for deoxygenation. S.C.C. = short-circuit current in microamperes per 7 cm^2; PD = transbladder potential difference in millivolts.

1×10^{-5} M, both the short-circuit current and the potential difference fell precipitously towards 0. Of considerable interest is the fact that the same response attended the addition of DNP to the anaerobic system (Fig. 1B).

The effects of DNP on sodium transport, lactate formation, and tissue ATP concentrations in an oxygenated environment are summarized in Table I. Net sodium transport was diminished; aerobic glycolysis was stimulated; and

TABLE I

EFFECTS OF DNP (1×10^{-5} M) ON AEROBIC SODIUM TRANSPORT, LACTATE PRODUCTION, AND ATP CONCENTRATION IN THE ISOLATED TURTLE BLADDER

No. of experiments	Conditions	Measurements		
		Na influx	Na efflux	Net transport
			μmole/hr/7 cm²	
3	O_2	7.58	2.01	5.57 (±1.88)
	O_2 + DNP	4.56	2.56	2.00 (±0.70)
			Lactate	
			μmole × 10⁻²/hr/7 cm²	
9	O_2		139.3 (±27.0)	
	O_2 + DNP		332.4 (±43.7)	
			ATP	
			μmole/g wet tissue	
10	O_2		0.33 (±0.16)	
	O_2 + DNP		0.25 (±0.08)	

The values for net sodium flux and lactate formation were obtained using hemibladders in the transport chamber (see text). The ATP concentrations were obtained using tissue segments as described in the text. Values in parentheses are standard errors of the mean.

ATP tissue concentrations were decreased modestly. The effects of DNP on the same parameters, in the absence of molecular oxygen, are presented in detail in Tables II and III.

Table II depicts the results of 5 bidirectional sodium flux studies. Control measurements were made under anaerobic conditions; then dinitrophenol was added to the serosal solution in a final concentration of 1×10^{-5} M. Sodium influx decreased markedly after the addition of DNP and in 4 of the 5 experiments sodium efflux increased modestly. In all instances, the two rates

approached each other. As a consequence, the mean value for the net flux diminished from 5.25 to 1.35 μmoles/hr/7 cm².

The values for lactate formation under anaerobic conditions before and after the addition of DNP are shown in Table III. The mean value for 8 experiments was 0.104 μmole/hr/7 cm²; the mean value for the same membranes following the addition of DNP was 0.245. Thus, the addition of DNP resulted in an approximately twofold increase in anaerobic glycolysis.

ATP tissue concentrations in bladder segments incubated anaerobically with and without DNP are also shown in Table III. In the absence of DNP,

TABLE II

THE EFFECTS OF DNP (1×10^{-5} M) ON ANAEROBIC SODIUM TRANSPORT

Experiment	Conditions	Influx	Efflux	Net
			μmole/hr/7 cm²	
1	N_2	3.53	0.44	3.09
	N_2 + DNP	1.78	1.72	0.06
2	N_2	3.99	0.70	3.29
	N_2 + DNP	1.76	1.43	0.33
3	N_2	5.70	1.22	4.48
	N_2 + DNP	1.26	1.08	0.18
4	N_2	6.52	0.12	6.40
	N_2 + DNP	2.92	1.38	1.54
5	N_2	9.26	0.27	8.99
	N_2 + DNP	7.00	2.36	4.64
	Mean N_2	5.80	0.55	5.25
	SE	(±1.00)	(±0.19)	(±1.10)
	Mean N_2 + DNP	2.94	1.59	1.35
	SE	(±1.05)	(±0.22)	(±0.87)

Studies were initiated after the membranes had equilibrated in oxygenated Ringer's solutions for at least 60 min (2).

the mean value (expressed in micromoles per gram of wet weight) was 0.355. The corresponding value for bladder segments from the same animals incubated in the presence of DNP was 0.264. The decrease averaged 25.6%.

In Table IV the rate of lactate formation is compared with the rate of glycogen utilization by the same tissue segments in the presence of DNP. The control value for glycogen content was determined on tissue immediately after removing the bladder from the turtle. The segments used for study were taken from the same bladder in each experiment and were incubated in Erlenmeyer flasks in 10 ml of Ringer's solution containing 1×10^{-5} M DNP and agitated in a Dubnoff metabolic shaker at room temperature (22° C) for 2 hr; 100% nitrogen was used as the gas phase. Lactate formation was measured in the supernatant Ringer solution. The values for glycogen are

expressed in terms of glucose residue. Theoretically each mole of glucose metabolized should yield 2 moles of lactate. The experimental data shown in Table IV reveal a relationship close to the theoretical one.

The possibility that oxidative metabolism might persist under the conditions of these experiments must be considered. This could result either from trace

TABLE III

EFFECTS OF DNP (1×10^{-5} M) ON ANAEROBIC LACTATE FORMATION AND ATP CONCENTRATIONS OF THE ISOLATED TURTLE BLADDER

No. of experiments	Lactate formation Conditions	
		μmole $\times$ 10^{-3}*/hr/7 cm²*
8	N_2	104.5 (±26.8)
8	N_2 + DNP	245.4 (±42.2)
	ATP concentration	
		μmole $\times$ *g wet weight*
10	N_2	0.355 (±0.09)
10	N_2 + DNP	0.264 (±0.03)

Lactate studies were performed using hemibladders in transport chambers; after equilibration in oxygenated Ringer's, 100% nitrogen was used as the gas phase. The measurements designated as N_2 were made during a period of approximately 60 min. The DNP then was added to the serosal solution and a 10 to 15 min period was allowed for the drug to penetrate the cell water. The measurements designated as N_2 + DNP were obtained during the ensuing hour.

ATP measurements were performed on tissue segments. All flasks were gassed with N_2. After 30 to 60 min of exposure to the anaerobic conditions, DNP was added to one of each pair of flasks and incubations were continued for an additional hour.

The values in parentheses are standard errors of the means.

quantities of oxygen in the proximity of the mitochondria, or from dismutation. In this event, the major energy-yielding reaction could be aerobic, rather than anaerobic, and the DNP effect could be a conventional one. To evaluate this, the following experiments were performed. Hemibladders were suspended in plastic chambers in the manner described above and after obtaining control measurements in oxygenated Ringer's the Ringer solutions were deoxygenated with 100% nitrogen. Iodoacetate (1×10^{-4} M) was added to inhibit glycolysis and pyruvate was added in a concentration of 1×10^{-2} M to provide a substrate for oxidative metabolism in the event that

electron acceptors were available in the system. Two experiments of this design are presented in Table V. In these experiments deoxygenation did not inhibit net sodium transport and the values for anaerobic net transport prior to the addition of the iodoacetate and pyruvate were slightly greater than the aerobic values. However, after addition of the iodoacetate, and despite

TABLE IV
CORRELATION BETWEEN GLYCOGEN UTILIZATION AND LACTATE FORMATION

Experiment	Glycogen utilized	Lactate formed
	μmole glucose/g	*μmole/g*
1	4.34	8.45
2	5.09	10.64
3	3.90	8.18
4	2.87	6.26
Mean	4.05	8.38

These studies were performed in the presence of 10^{-5} M 2,4-dinitrophenol under anaerobic conditions.

TABLE V
INHIBITION OF ANAEROBIC SODIUM TRANSPORT BY IODOACETATE: EFFECTS OF PYRUVATE

Conditions	Influx	Efflux	Net	S.C.C.
		μa/7 cm²/hr		
O_2	116.3	20.1	96.2	78.9
N_2	115.5	13.7	101.8	45.9
N_2 + IAA + pyruvate	88.7	26.3	62.4	11.1
	58.2	34.3	23.9	5.9
O_2	94.9	27.3	67.6	74.9
N_2	102.6	10.7	91.9	61.0
N_2 + IAA + pyruvate	34.3	38.6	−4.3	37.3
	35.9	19.0	16.9	28.0

S.C.C. = short-circuit current.
The details of these studies are described in the text. The iodoacetate was added to the serosal solution. The pyruvate was added in equimolar amounts to both mucosal and serosal solutions.

the presence of pyruvate, net transport fell strikingly in a manner similar to that observed with iodoacetate alone (4). The discrepancy between net sodium transport and the short-circuit current has been described previously (2).

Oligomycin shares in common with dinitrophenol the ability to interfere with energy conservation associated with mitochondrial oxidation. However, in contrast to dinitrophenol, oligomycin does not uncouple oxidation from

TABLE VI

EFFECTS OF OLIGOMYCIN (4 μG/ML AND 40 μG/ML FINAL CONCENTRATION) ON NET SODIUM TRANSPORT UNDER ANAEROBIC CONDITIONS

Experiment	Conditions	Oligomycin 4 μg/ml		Net	S.C.C.
		Influx	Efflux		
			μa/7cm²/hr		
1	O_2	186.3	63.0	123.3	136.0
		171.0	57.4	113.6	111.5
	N_2	196.2	78.5	117.7	62.8
	N_2 + oligomycin	121.9	54.9	67.0	12.4
		143.6	54.9	88.7	2.9
2	O_2	84.1	5.7	79.4	107.3
	N_2	65.7	6.8	64.9	59.3
	N_2 + oligomycin	74.8	8.0	66.8	16.3
3	O_2	144.9	26.4	118.5	
	N_2	139.4	29.5	109.9	52.4
	N_2 + oligomycin	89.8	42.6	47.2	10.5
4	O_2	148.1	42.6	105.5	125.9
	N_2	169.6	35.6	134.0	47.8
	N_2 + oligomycin	93.4	40.6	52.8	9.1

Experiment	Conditions	Oligomycin 40 μg/ml		Net	S.C.C.
		Influx	Efflux		
			μa/7cm²/hr		
1	O_2	79.6	2.7	76.9	125.9
	N_2	103.7	9.1	94.6	99.8
		92.7	27.1	65.6	37.9
	N_2 + oligomycin	77.2	3.2	74.0	16.6
		56.8	2.7	54.1	9.0
2	O_2	65.7	2.9	62.8	78.8
	N_2	61.4	8.8	52.6	66.3
		78.8	6.7	72.1	47.8
	N_2 + oligomycin	68.6	0.8	67.8	23.3
		53.9	4.3	49.6	9.2
3	O_2	59.2	0	59.2	79.4
	N_2	100.8	44.8	56.0	47.7
	N_2 + oligomycin	49.3	7.8	41.5	16.4
		50.1	3.2	46.9	4.1
4	O_2	61.1	16.9	44.2	87.9
	N_2	52.3	11.8	40.5	75.3
	N_2 + oligomycin	61.9	14.7	47.2	18.7
		47.7	12.9	34.8	7.6

phosphorylation. The relevance of using this compound in these experiments will be shown in further detail in the Discussion.

The effects of oligomycin are shown in Tables VI through VIII. Sodium transport (Table VI) was measured using two different concentrations of

TABLE VII

EFFECTS OF OLIGOMYCIN ON LACTATE FORMATION

Experiment	Aerobic conditions		Δ
	Control	Oligomycin	
	μmole × 10^{-3}/7 cm^2/*hr*		*per cent*
1	327	162	−50
2	38	105	+176
3	304	222	−27
4	133	130	−2
5	190	78	−59
6	76	76	±0
7	180	245	+36
8	186	293	+58
9	479	61	−87
Mean	213	153	+5
Experiment	Anaerobic conditions		Δ
	Control	Oligomycin	
	μmole × 10^{-3}/7 cm^2/*hr*		*per cent*
1	245	176	−28
2	568	54	−90
3	495	680	+37
4	192	150	−22
5	332	300	−10
6	171	201	+18
7	263	63	−76
8	178	32	−82
9	251	389	+55
10	489	238	−51
11	515	193	−63
12	73	79	+8
13	178	234	+31
14	808	478	−41
Mean	340	233	−22

oligomycin. In the upper portion of Table VI the effects of 4 μg oligomycin per ml of Ringer's solution are shown and in the lower portion of the table, the effects of 40 μg/ml are shown. Control measurements were performed first in the presence of oxygen and then under anaerobic conditions prior to the addition of the inhibitor. With 4 μg/ml, some decrease in net transport

occurred in 3 of the 4 experiments after oligomycin was added, but for the group the change was not impressive. With 40 μg/ml the changes were even less impressive and resembled those seen with consecutive measurements under conditions of sustained anaerobiosis (2).

Table VII depicts the influence of oligomycin (4 μg/ml) on aerobic and anaerobic lactate formation. In the aerobic experiment no consistent pattern

TABLE VIII

EFFECTS OF OLIGOMYCIN ON AEROBIC AND ANAEROBIC ATP CONCENTRATIONS IN ISOLATED TURTLE BLADDER

ATP: micromoles per gram of wet weight

Experiment	Aerobic		Δ
	O_2	O_2 + oligomycin	
			per cent
1	0.272	0.096	−65
2	0.192	0.112	−42
3	0.529	0.401	−24
4	0.256	0.096	−63
5	0.321	0.240	−25
Mean	0.321	0.192	−44

Experiment	Anaerobic		Δ
	N_2	N_2 + oligomycin	
			per cent
1	0.192	0.112	−42
2	0.256	0.096	−63
3	0.529	0.401	−24
4	0.321	0.240	−25
5	0.256	0.176	−31
6	0.256	0.192	−25
Mean	0.304	0.208	−35

The concentration of oligomycin used in these studies was 4 μg per ml of Ringer's solution.

emerged. Under anaerobic conditions, the results also were inconsistent. However, in 9 of 14 experiments a decrease was observed and in 7 experiments the decrement was appreciable.

In Table VIII, the tissue concentrations of ATP are shown for bladder segments incubated with and without oligomycin (4 μg/ml). Eleven experiments were performed, 5 under aerobic conditions and 6 under anaerobic conditions. In the flasks containing the oligomycin ATP concentrations were lower than in the control flasks in each instance and with both gas phases.

The mean percentage decrease was 44% in the aerobic studies and 35% in the anaerobic ones.

DISCUSSION

Dinitrophenol has been found to be a powerful inhibitor of anaerobic sodium transport by the isolated urinary bladder of the fresh water turtle. Anaerobic metabolism, however, was not inhibited. On the contrary lactate formation was markedly stimulated by DNP. The fact that a 1:2 molar ratio was observed between glycogen utilization (measured as glucose residue) and lactate formation, indicates that the glycolytic pathway was completed in the presence of DNP. Anaerobic metabolism, therefore, must have proceeded past both energy conservation sites of glycolysis.[1] Three moles of ATP, thus, should have been generated per mole of glucose (from glycogen) metabolized to lactate. Hence, ATP synthesis should have been increased by DNP by approximately twofold. On the other hand, sodium transport diminished strikingly and it has been shown previously that approximately half of the anaerobic glycolysis of the turtle bladder is coupled to active sodium transport (3). Accordingly, total ATP utilization should have been decreased appreciably (i.e. by as much as 50%). The net result should have been an increase in the tissue concentration of ATP. But the ATP stores were not increased; rather there was a modest decrease. Thus if ATP were formed during the DNP-stimulated glycolysis, it must have been dissipated without transferring its energy effectively to sodium transport. If ATP were not formed, DNP must have uncoupled glycolysis from phosphorylation.

Each of these two possibilities warrants detailed scrutiny; so also does the question as to why the residual ATP in the cells was not capable of sustaining sodium transport.

Increased ATP Degradation Rate DNP is known to stimulate mitochondrial ATPase activity (12–14). The possibility must be considered, therefore, that ATP synthesis did occur at an increased rate in the presence of DNP but that the newly formed ATP was dissipated by virtue of enhanced ATPase activity. This presumably would involve plasma membrane ATPase activity as well as mitochondrial ATPase activity in view of the fact that transcellular ion transport is a plasma membrane phenomenon. The ATPase reaction, in turn, could lead to increased glycolysis. Although this explanation must be seriously considered, it would not readily account for the almost total abolition of sodium transport that was observed in the presence of DNP when the residual ATP concentrations were approximately 75% of the control levels.[2]

[1] ATP synthesis occurs at two points in the glycolytic pathway. The first is with the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate; the second is with the conversion of phosphoenol pyruvate to pyruvate.

[2] We recently have found that the ATP concentration of the epithelial cell layer of the turtle bladder

An additional explanation also would be required for the failure of the energy released by the ATPase-induced hydrolysis of ATP to energize the sodium pump. Thus if accelerated ATP dissipation occurred as a direct result of DNP stimulation (or induction) of ATPase activity, additional mechanisms of action would be required to explain (*a*) the failure of the persisting ATP stores to serve as energy sources for sodium transport, and (*b*) the fate of the energy released during the hydrolysis of the newly formed ATP.

In an effort to clarify this question, a series of experiments was performed on bladder segments using iodoacetic acid (IAA) to block glycolysis. DNP then was added in association with the IAA. The results of these experiments are summarized in Table IX. ATP concentrations were markedly decreased with IAA alone and only modestly decreased with DNP alone. However, when both IAA and DNP were added, the decrement was greater than with IAA alone. Lactate formation shown in the same table was inhibited with IAA, stimulated with DNP, and when both drugs were used, the values were less than the control but were greater than with IAA alone. These data thus suggest that when anaerobic glycolysis is blocked, DNP does initiate a net ATPase effect. However, for reasons that will be considered below, this experiment is not conclusive and the results could be explained on the basis of an "operational" ATPase effect of DNP.

The Energy Leak Hypothesis If the accelerated rate of glycolysis induced by DNP is associated with a corresponding acceleration in the rate of production of ATP, then as indicated, there could be an "energy leak." This would be beyond the primary steps in glycolysis and would presumably involve the immediate source of energy for the sodium pump. Such an energy leak would allow for the net dephosphorylation of existing ATP stores and thus for an operational ATPase effect. The latter, in turn, could serve to stimulate glycolysis.

Among the possible mechanisms for an energy leak in the present experiments would be a conventional uncoupling of oxidation from phosphorylation by DNP. This would require that oxidative metabolism continue under conditions presumed to be anaerobic. As indicated previously this could occur by virtue of the presence of trace quantitities of molecular oxygen in the vicinity of the mitochondria and/or by dismutation. If there were electron acceptors available in the system, dinitrophenol could act in a conventional manner on a high energy intermediate of oxidative phosphorylation. Several considerations make this explanation unlikely. (*a*) The almost complete inhibition of sodium transport produced by DNP would require that all of the energy in the anaerobic studies be derived from oxidative pathways, yet glycolysis (and glycolytic ATP production) are markedly increased when the

is approximately the same as that of the whole bladder and incubation of the isolated epithelial sheets in the presence of DNP also results in a modest decrease in ATP levels (15).

gas phase is changed from O_2 to N_2. Furthermore, as noted earlier, there is a stoichiometric relationship between anaerobic glycolysis and anaerobic sodium transport. (*b*) The presence of electron acceptors should allow for continued energy transformation despite the inhibition of glycolysis if the appropriate substrate is provided. Yet when glycolysis was inhibited by iodoacetate in the anaerobic system, the addition of pyruvate did not sustain

TABLE IX

EFFECTS OF IAA AND DNP ON LACTATE FORMATION AND ATP CONCENTRATION OF ISOLATED TURTLE BLADDER

Experiment	ATP			
	N_2	N_2 + IAA	N_2 + DNP	N_2 + IAA + DNP
	μmole/g wet weight			
1	0.386	0.011	0.197	0.004
2	0.325	0.020	0.227	0.004
3	0.255	0.025	0.237	0.016
4	0.242	0.019	0.186	0.006
5	0.264	0.020	0.086	0.016
6	0.264	0.012	0.163	0.189
Mean	0.290	0.019	0.182	0.009
Experiment	Lactate production			
	N_2	N_2 + IAA	N_2 + DNP	N_2 + IAA + DNP
	μmole × 10^{-3}*/mg wet weight/hr*			
1	0.55	0	0.76	0.26
2	0.56	0	0.95	0.30
3	0.72	0	1.66	0.18
4	0.54	0	0.73	0.20
5	0.56	0.23	0.76	0.46
6	0.27	0	0.38	0
Mean	0.53	0.04	0.87	0.23

In each experiment tissue from one bladder was divided into segments and distributed equally among four incubation flasks.

sodium transport. (*c*) In previous studies, neither KCN nor antimycin A (2, 4) both of which block forward electron transport, abolished sodium transport.

A second explanation for the DNP effect also would involve the conventional hydrolysis of a high energy intermediate of oxidative phosphorylation. However, no electron acceptor would be required. This is shown schematically in Fig. 2A. ATP is synthesized *via* glycolysis and the newly formed ATP enters a mitochondrion. (The entire sequence might also occur in the cytoplasm.) Thereafter the normal oxidative pathway of ATP formation is reversed and the

high energy intermediates appear in reverse order.[3] DNP then could effect the hydrolysis of a nonphosphorylated high energy intermediate (shown in the diagram as ~1) thereby creating an energy leak and diverting the energy from the sodium pump. In this view the DNP-sensitive intermediate, rather than ATP, would be the immediate source of energy for sodium transport.[4] The oligomycin studies were performed in an effort to examine this hypothesis. Oligomycin is thought to act between the site of action of DNP and ATP (18, 19). It is generally believed that the oligomycin acts at a phosphorylated intermediate step (19) which in the diagram in Fig. 2 is represented as ~2. With a reversal of the normal pathway for mitochondria-linked ATP synthesis, oligomycin might block the formation of ~1, the DNP-sensitive

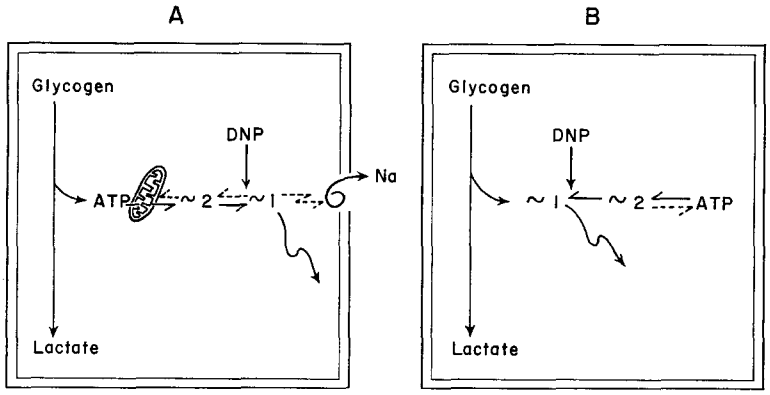

FIGURE 2. The details of these models are presented and fully discussed in the text. Fig. 2A depicts the formation of ATP from glycolysis and the subsequent reversal of the normal pathway of high energy intermediate synthesis. This reversal would involve the use of oxidative machinery. Fig. 2B depicts the formation of high energy intermediates directly from glycolysis.

intermediate and the presumed source of energy for the sodium pump. If oligomycin, therefore, were to act in this manner in the turtle bladder, it should inhibit anaerobic sodium transport, if the schema in Fig. 2A were valid. However, sodium transport was not affected appreciably by the oligomycin in concentrations as high as 40 μg/ml.

The final type of energy leak that must be considered involves the inactivation by DNP of a high energy intermediate derived from glycolysis *per se* (Fig. 2B). An essential correlary of this hypothesis is that the intermediate rather than ATP would serve as the energy source for the sodium pump. DNP then would serve to uncouple glycolysis from phosphorylation and would

[3] This concept has been presented in greater depth by Van Rossum (16).

[4] Slater (17) first suggested in 1953 that an intermediate rather than ATP could be the energy source for energy-requiring processes such as ion transport.

therefore have an anaerobic effect which simulates the classic aerobic effect. This explanation is not entirely without conceptual precedent, for Lipmann (20) in a discussion of a paper by Racker (21) in 1959 noted that the growth of anaerobic cells (e.g. yeast) can be inhibited by DNP and suggested that there might be a common pathway for both aerobic and anaerobic energy supply.

If an intermediate, or intermediates, of glycolysis exist in the fresh water turtle bladder, DNP might affect their hydrolysis in a manner analogous to that postulated to occur in oxidative metabolism (5–7). This would constitute an energy leak; it also could initiate an operational ATPase effect associated with a net dephosphorylation of ATP and a dissipation of some portion of the cellular stores of ATP. The decrease in ATP (or the attendant increase in ADP) might serve as the stimulus to glycolysis accounting for the increment observed in these experiments. Oligomycin would act distal to the DNP-sensitive intermediate (i.e. between the intermediate and ATP or at ~2 in Fig. 2B) and it therefore would not block sodium transport. On the other hand oligomycin might block synthesis of new ATP and some dissipation might occur by virtue of mitochondrial and possibly plasma membrane ATPase activity. Finally this mechanism of action of DNP could provide an explanation for the complete, or virtually complete, inhibition of sodium transport that occurred in the presence of relatively high tissue ATP concentrations. Thus, if a DNP-sensitive intermediate, rather than ATP, were the immediate source of energy for the pump, the ATP stores within the cells would not sustain sodium transport.

The present data do not exclude a direct inactivation of a sodium carrier by DNP. However, if there were such an effect, it would not explain the imbalance between glycolysis and transport (i.e. between the increase in energy production and the decrease in energy utilization for transport). Obviously DNP could have two or more separate effects and thus it could inhibit the carrier directly and at the same time activate ATPase with the latter serving to stimulate glycolysis.

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