membrane, has been developed from studies of calcium accumulation in the presence of permeant and impermeant anions.

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*INDUCED ACTIVE TRANSPORT OF IONS IN MITOCHONDRIA**

BY BERTON C. PRESSMAN

JOHNSON RESEARCH FOUNDATION, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

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The antibiotic valinomycin has been shown to have the property of inducing the active transport of monovalent ions (K^+, Rb^+, Cs) in mitochondria.[1] This communication extends the list of substances, both natural and synthetic, which share this property, and examines the dependence of induction activity of molecular structure. We also wish to report on the instrumentation we have assembled

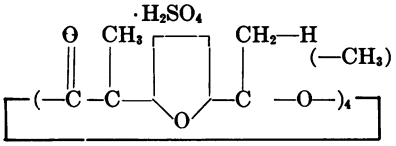
for extending this investigation and to indicate some of the preliminary results obtained.

Methods and Materials.—The data in Table 1 were based on the concentration of a given compound required to elicit a standard initial rate of H^+ ejection (1 mM H^+/min) from rat liver mitochondria prepared by the method of Schneider.[2] Since the titrations carried out required several mitochondrial preparations, each value in the table has been normalized by reference to the action of a valinomycin standard on the same mitochondrial preparation used for the particular assay.

In the experimental figure (Fig. 1), light scattering was monitored at 540 mμ with the Eppendorf photometer equipped with a 90° light-scattering accessory. A linear relationship between mitochondrial concentration and light scattering was maintained by confining the light path through the corner of the 2.5-cm^2 reaction cuvette to 0.4 cm. Na^+, K^+, and H^+ were monitored with Beckman electrodes 39046, 39047, and the A. H. Thomas 4858-L15 combination electrode, respectively. The glass electrodes, along with the Ag-AgCl reference of the combination electrode used in common, were connected to either Beckman model 76 or Radiometer model 22 pH meters. The outputs of the pH meters and Eppendorf, as well as that of the Radiometer E-5044 Clark-type electrode used to monitor O_2, were fed to amplifiers specially designed to drive the low impedance galvanometers of the Consolidated Electrodynamics model 5-124 recording oscillograph without cross interaction. All electrodes were mounted in the cuvette from above and stirring was accomplished with a magnetic stirrer from below. Accumulation of ions by mitochondria was measured indirectly from the extramitochondrial changes sensed by the glass elec-

TABLE 1

ION TRANSPORT INDUCTION PROPERTIES OF VARIOUS AGENTS

	Structure	Compound	Mol. wt.	Ring atoms	Relative activity
1.	⌐(D-Val-L-Lac-L-Val-D-Hyval)$_3$¬	Valinomycin	1111	36	100
2.	⌐(D-Val-L-Lac-L-Val-D-Hyval)$_2$¬ L-Val-L-Lac-L-Val-D-Hyval¬		1111	36	13
3.	⌐(D-Val-L-Lac-L-Val-D-Hyval)$_2$¬ D-Val-D-Lac-L-Val-D-Hyval¬		1111	36	2.2
4.	⌐(L-Val-L-Lac-L-Val-D-Hyval)$_3$¬		1111	36	0.00
5.	⌐(D-Val-L-Lac-L-Val-D-Hyval)$_2$¬		741	24	0.00
6.	2 Gly; 4 L-Ala; 8 D-Leu; 4 D-Val; 4 L-Val; 8 L-Try; 2 Ethanolamine	Gramicidin A	3725	?	3.3
7.	" " + L-Phe	Gramicidin B	3725	?	6.0
8.	" " + L-Tyr	Gramicidin C	3725	?	2.5
9.	⌐(L-Val-L-Orn-L-Leu -D-Phe-L-Pro)$_2$¬	Gramicidin S	1240	30	0.00
10.		Nonactin			
		Squibb SQ 15859	750	28	0.34
11.	"	Squibb EJB-V-14	750	28	0.88

The complete test system consisted of TRIS-glutamate, pH 7.4, 3 mM; TRIS-malate, pH 7.4, 3 mM; TRIS-Cl, pH 7.4, 20 mM; KCl, 15 mM; sucrose, 240 mM; final volume 5 ml. The reaction vessel (2.5 cm diameter) was jacketed at 25° and stirred with a magnetic bar. After initial equilibrium of the medium with Beckman electrodes no. 41252 (glass) and no. 41239 (calomel), rat liver mitochondria were added (final concentration 4 mg protein/ml), re-equilibration permitted (*ca.* 2 min) and the test agent dissolved in EtOH (<0.1 ml) added. Acid liberated was followed with a Beckman model 76 pH meter connected to a 10-mv potentiometric recorder. Assay comparisons were conducted as described in text under *Methods*. In addition to standard abbreviations for amino acids, the following have been used: Lac, lactate; Hyval, hydroxyisovalerate.

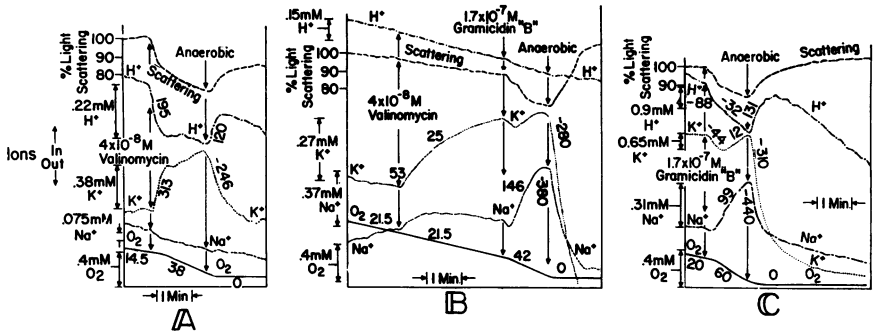


FIG. 1.—*Experiment A:* Valinomycin-induced uptake of K^+. Concentrations of all reactants the same as in Table 1 except for 1.9 mM NaCl supplement, final volume 10 ml, and temperature 22°. The medium was equilibrated against 100% O_2 before the addition of mitochondria. Two min before addition of 4×10^{-8} *M* valinomycin, 20 γ oligomycin were added. Figures on tracings represent slopes, expressed in μmole/gm protein/min. *Experiment B:* Valinomycin-induced uptake of "leaked" K^+ and subsequent gramicidin-induced uptake of Na^+. System contained TRIS glutamate, 3 mM; TRIS malate, 3 mM, TRIS-Cl, pH 7.4, 20 mM, NaCl, 1.9 mM; choline chloride, 13 mM, phosphate (TRIS), pH 7.4, 1.25 *M;* sucrose, 240 mM. Oligomycin (20 γ) added 2 min before valinomycin. *Experiment C:* Gramicidin-induced uptake of Na^+. System the same as in *Experiment B* except for omission of phosphate and valinomycin.

trodes which were calibrated by the subsequent addition of known standards to produce excursions similar to those observed experimentally.

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Results.—Structure-activity correlations: Valinomycin (compound 1)[3] remains the most potent of the series tested. The concentration required to produce a standard response in these experiments was *ca.* 4×10^{-8} *M;* effects could easily be observed at less than one tenth this level. Inverting the optical configuration of a single D-valine (compound 2) or a single L-lactate (compound 3) reduces activity to 13 per cent and 2.2 per cent, respectively. Inverting all three D-valines (compound 4) causes the complete loss of detectable activity, as does reducing the ring size by eliminating a single sequence of four residues (compound 5).[4]

Of a series of uncoupling agents tested, gramicidin D alone has been found to share with valinomycin the ability to cause an ejection of H^+ from mitochondria.[5] Although the precise structure of gramicidin D is still unknown,[6] the commercial product consists of three components: *A* (compound 6), *B* (compound 7), and *C* (compound 8).[7] All have similar ion transport induction properties, although the "*B*" is appreciably more active than the others. The same organism producing gramicidin D also synthesizes gramicidin S (compound 9)[6] which is inactive.

Two macrotetrolide[8] samples, 19 and 20, differing in proportion of nonactinic-homononactinic residues, are also active. Other surface-active antibiotics tested, including amphiotericin B and polymixin B, showed no ion transport induction activity, nor did oleate, a representative chemical surface active agent. The polypeptide hormones bradykinin and angiotension were also inactive.

Cation electrode studies: We had previously reported that the ion specificity of valinomycin-induced transport (K^+, Rb^+, Cs^+; but not Li^+ or Na^+) was not

as marked in the case of gramicidin.[9] This fact is most clearly revealed by means of the ion-specific glass electrodes whose applicability to this type of study has already been demonstrated.[1]

Experiment *A* characterizes the K^+ uptake induced by valinomycin under the standard assay conditions of Table 1, and confirms that under these conditions H^+ ejection correlates with K^+ uptake. The half-time response of the H^+ and 39047 electrodes, *ca.* 2 sec, was due to the response time of the amplifiers. This validates the calculation of rates summarized in Table 2 for those portions of the tracings sustaining linearity over 5 sec. The maximum rate of K^+ accumulation recorded, 313 μmole/gm protein/min, under comparable conditions (substrate energized, phosphate absent) is surpassed only by Ca^{++} (rate over 1000),[10] the most rapidly transported divalent ion.[11] Maximum K^+ uptake, 95 μmole/gm protein, compares favorably with maximum Ca^{++} uptake under these conditions before irreversible swelling occurs.[12]

Since the half-time response of the oxygen electrode was 3 sec, the lag in respiratory stimulation following the initiation of K^+ transport, *ca.* 20 sec, is therefore unquestionably significant. At the initial stages of transport, respiration remains at the State 4 rate, 14.5 μmole/O_2/gm protein/min, ratio of 21.6. Since it is unlikely that all of the State 4 respiration can give rise to energy for ion transport, the calculated K^+/O_2 ratio, 21.6, is probably underestimated. Assuming a ~P/O ratio for glutamate-malate of 3, this corresponds to a K^+/~P ratio $\geqslant$ 3.6.

The possibility exists that an energy debt could pile up and the subsequent accelerated respiratory rate, or its increment over the State 4 rate, should be used for calculating the K/~P efficiencies. The duration of the accelerated respiration does not conform with the period of active K^+ uptake, as is the case with Ca^{++} uptake,[13] and appears to be related to the net accumulation of K^+ within the mitochondria rather than the rate of K^+ entry. As the gradient opposing K^+ uptake builds up, while the rate of uptake is maintained, the energy demand of further uptake increases, causing the gradual increase in respiration. Thus, in Experiment *A* the K/O_2 ratio falls from an initial value of 21.6 to 8.2. K^+

TABLE 2

SUMMARY OF QUANTITATIVE MEASUREMENTS FROM EXPERIMENTAL FIGURE

Experiment:	*A*	*B*		*C*	
Inducer:	Valinomycin	Valinomycin	Gramicidin	Gramicidin	
Cation:	K^+	K^+	Na^+	K^+	Na^+
Net transport induced (μmole/gm protein)					
Cation	95	68	92	−16	78
H^+	−55	0	−5	−22	
Cation/-H^+	0.58	0	0.06		0.28
Rates (μmole/gm protein/min)					
Cation (induced)	313	53	146	−44	99
Cation (anaerobic)	−246	−280	−380	−310	−440
H^+ (induced)	−195	0		−88	
H^+ (anaerobic)	120	0		131	
O_2 (base rate)	14.5	21.5		20	
O_2 (max after induction)	38	21.5	42	60	
Cation/O_2, base	21.6	2.5			
Cation/O_2, max	8.2	2.5	3.5		1.3
Cation/O_2, max-base	13.3	0	7.1		2.5

For experimental conditions, see experimental figure (Fig. 1). Entries have been omitted when considered of doubtful significance.

efflux also rises with the gradient. At the point that efflux balances influx and net K^+ uptake is maximal and stable, the steady state flux continues to dissipate considerable energy, thus maintaining the elevated respiratory rate. Some estimation of the magnitude of the steady state flux is revealed by the rates of cation efflux evoked by anaerobiosis, which in Table 2 can even exceed the maximal rate of net cation uptake. Cutting off access to energy with the uncoupling agent dinitrophenol leads to similar maximum efflux rates. Thus the energy required to initiate K^+ influx is small, and can be provided with a minimum of respiratory stimulation.

In Experiment *B*, mitochondria were incubated in a low Na^+ medium devoid of added K^+. Upon addition of valinomycin, both ion electrodes record the disappearance of ions from the medium. The uptake of 0.27 mM K^+ represents the return of K^+ leaked out of the mitochondria. The data of Berger[14] and Gamble[15] indicate that fresh mitochondria contain 100–150 μmole K^+/gm protein, which is subject to release on washing.[14] This corresponds to an average concentration in the reaction mixture of Experiment *B* of 0.4–0.6 mM K^+. The net K^+ translocation triggered by valinomycin is appreciable compared to that translocated in Experiment *A*, but the transport is much slower and is not accompanied by swelling, H^+ ejection, or respiratory stimulation, supporting the contention that factors other than net transport, such as flux, can exert dominant effects on the transport-associated mitochondrial activities. The small excursion of the Na^+ electrode following valinomycin addition is largely due to its partial sensitivity to the K^+ shift and does not represent any appreciable true Na^{++} movement.

After permitting the valinomycin-induced K^+ influx 3 min to approach equilibrium, gramicidin B was added. Although the swelling and respiratory stimulation previously associated with ion uptake ensue immediately, K^+, Na, and H^+ are all initially expelled by the mitochondria. Twelve sec after the gramicidin addition, Na^+ uptake commences, but uptake of K^+ is delayed by an additional 10 sec. Upon the approach of anaerobiosis, the ejection of K^+ occurs first, followed, as O_2 exhaustion proceeds, by Na^+ ejection, H^+ uptake, and mitochondrial contraction. K^+ ejection goes beyond the level before valinomycin addition, due to the release of some K^+ originally within the mitochondrial preparation. The anaerobic contraction also goes beyond the initial state of the mitochondria, a phenomenon dependent on the presence of phosphate omitted in Figure 1, *A* and *C*. The phosphate was included in this experiment, as it enhances slightly the K^+ uptake.

The conditions of Experiment *C* differed from Experiment *B* only in that neither phosphate nor valinomycin was added prior to the gramicidin. The results establish that gramicidin-induced Na^+ transport does not require the presence of valinomycin. The time lag between Na^+ and K^+ influx exceeds that seen in Experiment *B*; *K^+ ejection* continues 15 sec after the onset of *Na^+ uptake*. H^+ ejection, swelling, and respiratory stimulation all occur during the Na^+–K^+ expulsion phase; during the Na^+–K^+ uptake phase, H^+ ejection and swelling continue at reduced rates.

Discussion.—The data of Table 1 establish that ring size, and sequence of D and L configurations are all critical in determining the ion transport induction properties of a molecule.

Several of the naturally occurring compounds of Table 1 have been grouped

together as "surface-active antibiotics."[16] However, the profound effects exerted by minimal alterations of structure, the remarkably low concentrations at which valinomycin in particular is active, and the lack of induction effects by other surface active agents (oleate, polymixin B, amphotericin B) establish that surface activity *per se* is not the chief molecular attribute behind these properties. The data are more consistent with a structurally specific relationship between the active inducers and a specialized mitochondrial receptor site.

Chappell has suggested that the differences in Na^+–K^+ specificity between valinomycin- and gramicidin-induced transport resides in the size of the hole in the ring of the inducer molecule, which in turn determines the size of a flaw produced in a membrane surrounding the mitochondrial ion pump.[9, 17] It was proposed that the ring of the cyclo*octa*depsipeptide valinomycin would permit the passage of K^+ (hydrated ionic ratios 2.32 Å)[18] but not Na^+ (2.76 Å),[18] while the larger ring of the cyclodecapeptide gramicidin would pass both ions as well as Li^+ (3.40 Å).[18] The structure of valinomycin is actually a cyclo*dodeca*depsipeptide,[19] however, and that mistakenly presented for gramicidin D[9, 17] was actually that of the inactive gramicidin S. A simple "membrane flaw" mechanism opening access to a nonspecific H^+ pump is also inconsistent with the H^+ ejection accompanying the transient Na^+ and K^+ ejection immediately following the gramicidin B addition in Experiment *C*, or the subsequent transient countermovement of Na^+ and K^+ in Experiments *B* and *C*. The fact that either the uptake or ejection of Na^+ and K^+ can accompany H^+ ejection also opposes the further suggestion that alkali ion transport is driven by a respiration-dependent H^+ pump.[20]

Our studies have indicated that all of the active compounds in Table 1 can induce some degree of Na^+ dependent H^+ ejection, although higher concentrations of Na^+ than K^+ may be required. Discrimination between Na^+ (or Li^+) and K^+ (or Rb^+, Cs^+) in the transport induced remains greatest for valinomycin. The factors determining the Na^+–K^+ specificity of induced transport reside directly in some aspect of the interaction of the inducer with the mitochondrial receptor site and are not yet clear.

Mitochondrial swelling was implicated as a significant parameter for characterizing induced ion transport by the observation that, unlike most uncoupling agents, gramicidin D produced a KCl-dependent swelling of mitochondria.[21] Chappell and Crofts have suggested that mitochondrial swelling during ion transport might be a passive osmotic consequence of the induced active accumulation of ions within the mitochondria.[20] A similar position has been taken by Rasmussen *et al.* to explain the swelling accompanying parathyroid-induced K^+ transport.[22] We view mitochondrial swelling and ion transport as mechanistically associated but not inextricably so. Thus in Experiment *B*, K^+ accumulation follows valinomycin addition without swelling, while in Experiment *C* swelling accompanies the initial *release* of ions from mitochondria.

Even when swelling and transport occur together, as in Experiment *A*, the swelling is more than could be accounted for by a simple osmotic process. The movement of 0.38 mM K^+ is counterbalanced by an ejection of 0.22 mM H^+, for a net ionic shift of 0.16 mM. Since the osmotic equivalent of KCl is 154 mM, this shift corresponds to a volume of 0.16/154 or 1.04 ml/liter. One gram equivalent of rat liver mitochondria pack to 6.6 ml,[23] which, assuming 20 per cent interstitial

fluid, equals 5.3 ml true volume. Thus the 4 mg/ml mitochondrial protein level represents a volume of 21.2 ml/liter. The predicted mitochondrial volume increase would be 1.02/21.2 or 4.9 per cent. Since Koch reported that optical density, which is linearly proportional to 90° scattering,[24] varies inversely as the $^2/_3$ power of mitochondrial volume,[25] the predicted decrease in scattering would be 2.9 per cent. The observed value in Experiment *A* of 20 per cent is therefore inconsistent with the proposed osmotic mechanism. The discrepancy is still greater if part of the translocated K^+ becomes mitochondrially bound and therefore osmotically inactive.

Both induced transport and mitochondrial swelling and contraction are reversible processes that involve the transduction of chemical bond energy into mechanical work at the level of either ions (active transport) or structural elements (contractile shifts). We believe these phenomena are coupled by either the sharing of several metabolic steps in common, or the structural relationship of the contractile element and the ion pump, concurring with the concept of a metabolically linked mechano-enzyme system in mitochondria.[26, 27] Those situations in which ion transport fails to correlate with swelling could be explained if the latter complies more closely with ion flux than with net ionic displacement.

The value 3.6 for the $K^+/\sim P$ ratio, minimal for the efficiency of K^+ transport by rat liver mitochondria, compares favorably to values of 3 for Na^+ transport in erythrocytes[28] and 8 for Na^+ transport in the herring gull salt gland (latter value calculation based on State 3 respiratory rate).[29]

Independent estimations of the $Ca^{++}/\sim P$ mitochondrial transport efficiency agree at a value close to 2.[11, 13, 14] This would require that, if the monovalent and divalent transport mechanisms are analogous, transport efficiency is determined by the number of charge equivalents rather than ionic equivalents translocated. Alternatively, the cation/$\sim$P ratio for monovalent ions could be nonintegral and capable of varying as does the gradient against which the ions move. Table 2 does indicate considerable variation of cation/O_2 ratios under different experimental conditions.

Induced ion transport appears a rather general phenomenon phylogenetically, since all mitochondria tested by us (rat liver, pigeon heart, rabbit kidney,[30] potato tuber[31]) show a K^+ and energy-dependent valinomycin-induced H^+ ejection. The antibiotic properties of the inducers further suggest to us that they act similarly at the bacterial level, introducing lethal aberrations into the energy metabolism of susceptible organisms.

The orientation of mitochondria with respect to cellular duct networks in organs specialized for ion transport, such as the gull salt gland[32] and the mosquito larval anal papillae,[33] indicate that mitochondrial transport activity may be utilized for transport at the cellular and tissue level. Mitochondrial transport activity might also regulate energy transfer directly, since under certain conditions mitochondrial oxidative phosphorylation becomes completely dependent on added K^+.[34]

The extreme sensitivity of mitochondria to the inducers studied here supports the likelihood of a purposeful receptor site for control of monovalent ion transport, but raises the question what the natural activators of such a site in higher organisms could be. In this regard, the report of K^+ transport induction activity for parathyroid hormone by Rasmussen *et al.* might prove significant.[22]

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