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The Action of Certain Antibiotics on Mitochondrial, Erythrocyte and Artificial Phospholipid Membranes

THE ROLE OF INDUCED PROTON PERMEABILITY

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1. The action of the antibiotics enniatin A, valinomycin, the actin homologues, gramicidin, nigericin and dianemycin on mitochondria, erythrocytes and smectic mesophases of lecithin–dicetyl hydrogen phosphate was studied. 2. These antibiotics induced permeability to alkali-metal cations on all three membrane systems. 3. The ion specificity on each membrane system was the same. 4. Enniatin A, valinomycin and the actins did not induce permeability to protons, whereas nigericin and dianemycin rendered all three membrane systems freely permeable to protons. 5. Several differences were noted between permeability induced by nigericin and that induced by gramicidin. 6. The action of all these antibiotics on mitochondrial respiration could be accounted for by changes in passive ion permeability of the mitochondrial membrane similar to those induced in erythrocytes and phospholipid membranes, if it is assumed that a membrane potential is present in respiring mitochondria.

The effects of valinomycin on mitochondrial respiration have been investigated extensively (McMurray & Begg, 1959; Pressman, 1963, 1965; Moore & Pressman, 1964; Höfer & Pressman, 1966; Harris, Höfer & Pressman, 1967*a*; Harris, Catlin & Pressman, 1967*b*). Gramicidin (Chappell & Crofts, 1965, 1966; Harris *et al.* 1967*a*), the actin homologues (Graven, Lardy, Johnson & Rutter, 1966*b*; Graven, Lardy & Rutter, 1966*c*; Graven, Lardy & Estrada-O, 1967; Harris *et al.* 1967*a*) and enniatin (Lardy, Graven & Estrada-O, 1967) have essentially similar effects, but differ in their alkali-metal cation requirements. Possible mechanisms for the action of these compounds have been discussed by Chappell & Crofts (1965) and by Mitchell (1966) in terms of an alteration of passive cation permeability activating a proton pump in the mitochondrial membrane. Alternatively, Lardy *et al.* (1967) have postulated that valinomycin, gramicidin and the actin homologues directly activate a specific cation pump in the membrane, whereas Pressman, Harris, Jagger & Johnson (1967) considered that the antibiotics induce cation permeability in lipid membranes but that in mitochondria 'the valinomycin group interacts preferentially at the ion pump assembly'.

In this Laboratory, model systems have been used to elucidate further the mode of action of these antibiotics. Gramicidin induced K^+–H^+ or K^+–Na^+ exchange in erythrocytes (Chappell & Crofts, 1966), but with nonactin and valinomycin no K^+–H^+ exchange occurred until an uncoupler of oxidative phosphorylation, e.g. 2,4-dinitrophenol or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, was added (Chappell, Henderson, McGivan & Robinson, 1968). At the suggestion of Dr A. D. Bangham smectic mesophases of lecithin–dicetyl hydrogen phosphate consisting of concentric bimolecular membranes intercalated with aqueous salt-filled compartments (Bangham, Standish & Watkins, 1965) were used as a model membrane system. Gramicidin was found to induce a K^+–H^+ exchange across these membranes. Valinomycin induced K^+–K^+ exchange, but no K^+–H^+ exchange occurred in the absence of an uncoupling agent (Chappell & Haarhoff, 1967; Chappell *et al.* 1968). More recently, the action of some or all of these antibiotics on single bilayer membranes of the type introduced by Mueller, Rudin, Tien & Westcott (1962) has been investigated (Mueller & Rudin, 1967; Lev & Buzhinsky, 1967; Andreoli, Tieffenberg & Tosteson, 1967). The effect of certain antibiotics on ascites-tumour cells (Levinson, 1967) and *Streptococcus faecalis* (Harold & Baarda, 1967) has also been noted.

Lardy and his co-workers have investigated the effects of nigericin and dianemycin on mitochondria (Lardy, Johnson & McMurray, 1958; Graven, Estrada-O & Lardy, 1966*a*; Estrada-O, Graven & Lardy, 1967*a*,*b*). These compounds reversed the

stimulation of respiration and cation uptake induced by valinomycin or the actins; the extent of inhibition depended on the substrate used, and the concentrations of K^+, phosphate and substrate. With erythrocytes (Henderson & Chappell, 1967; Pressman *et al.* 1967) and artificial phospholipid membranes (Henderson & Chappell, 1967), nigericin and dianemycin induced a rapid cation–H^+ exchange. Shavit & San Pietro (1967) found that nigericin produced K^+-dependent uncoupling in chloroplasts, and nigericin was also reported to inhibit the light-induced H^+ uptake (Packer, 1967).

In the present paper the cation and H^+ permeability induced in erythrocytes, smectic mesophases and mitochondria by these antibiotics is reported. It is concluded that passive permeability effects, similar to those induced in model systems, may be used to account for the action of these antibiotics on mitochondrial metabolism if it is assumed that there is an electrical potential across the inner mitochondrial membrane as postulated in the chemiosmotic hypothesis (Mitchell, 1966).

METHODS AND MATERIALS

Isotope exchange across artificial phospholipid membranes. Smectic mesophases were formed by shaking a mixture of lecithin + 10% dicetyl hydrogen phosphate in 145mM-^{134}CsCl, -^{86}RbCl, -^{42}KCl or -^{22}NaCl essentially as described by Bangham *et al.* (1965). The lipid was separated from untrapped tracer salt by passing 1ml. of the dispersion down a 27cm. × 1cm. column of Sephadex G-50 (coarse grade) equilibrated in the corresponding non-radioactive alkali-metal chloride solution. Portions (1ml.) of the dispersion emerging from the column were placed in stoppered dialysis bags and dialysed for 10min. periods against 5ml. of unlabelled salt solution. At the end of each 10min. period the dialysis bag was quickly washed and transferred into another 5ml. of salt solution. All solutions were buffered at pH7·0 with 3mM-tris phosphate. Antibiotics were added in a small volume of ethanol. The radioactive isotope appearing in the solution outside the dialysis bag in each 10min. period was estimated by plating out 0·5ml. samples and counting for radioactivity.

Uptake of radioactive isotopes into erythrocytes. Approx. 7ml. of human venous blood treated with heparin was diluted into an equal volume of 150mM-choline chloride containing 3mM-tris chloride, pH7·3. The erythrocytes were sedimented by centrifugation and washed twice in 4vol. of buffered iso-osmotic choline chloride. Samples of the packed-cell suspension were added to iso-osmotic buffered salt solutions at 30° containing radioactive cation as indicated in the legends to the Figures. Samples (1ml.) of the stirred suspension were taken at intervals and the cells separated by rapid centrifugation. Samples (25μl.) of the sedimented cells were lysed in 0·3ml. (approx.) of 0·1% cetyltrimethylammonium bromide solution contained in aluminium planchets, dried and assayed for radioactive disintegrations. The final samples contained at least 10 times the background level of radioactivity.

Radioactive isotopes were counted in the Nuclear-Chicago gas-flow system and disintegrations/min. converted into μmoles of cation from the specific radioactivity determined for samples of the suspension media. Corrections for the rapid decay of $^{42}K^+$ were made by using a simple programme designed for the Elliot 503 computer.

Volume changes in a smectic mesophase. Bangham, de Gier & Greville (1967) have established the relationship between E_{450} and volume of the hydrated liquid crystals of phospholipid contained in a smectic mesophase. The salt compositions of the smectic mesophases are described in the legends to the Figures; a Hilger–Gilford recording spectrophotometer was used to observe their changes of E_{450}.

pH *and* K^+ *movement.* Continuous measurements of pH and K^+ were made with EIL microelectrodes type GM23B and GM23B (BH115 glass) respectively. In some experiments a GM23B (BH104 glass) electrode was utilized to measure Na^+. A remote calomel reference electrode was used with a liquid junction to eliminate diffusion of K^+ from the reference into the electrode cell. The electrodes were calibrated with standard solutions of HCl, KCl or $KHSO_4$.

Materials. Egg lecithin was extracted and purified as described by Dawson (1963). Dicetyl sodium phosphate was obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.), and dicetyl hydrogen phosphate was extracted into chloroform from a suspension of this sodium salt in HCl. Other reagents used were of A.R. grade.

Rat liver mitochondria were isolated as described by Chappell & Crofts (1965) in a medium containing 0·25M-sucrose, 4mM-tris chloride and 1mM-ethylenedioxybis-(ethyleneamino)tetra-acetate, pH7·4.

Radioactive compounds were obtained from The Radiochemical Centre (Amersham, Bucks.). Antibiotics were kindly given by the following: monactin, a monactin–nonactin mixture and enniatin A by Dr W. Keller-Schierlein (Eidg. Technische Hochschule, Zürich, Switzerland); dianemycin and monensin by Dr R. L. Hamill (Eli Lilly and Co., Indianapolis, Ind., U.S.A.); nigericin by Dr R. L. Harned (Commercial Solvents Corp., Terre Haute, Ind., U.S.A.); valinomycin by Dr G. C. McDonald, Saskatoon, Sask., Canada; compounds X-206, X-464 and X-537A by F. Hoffmann–La Roche and Co., Basle, Switzerland; gramicidin D, a commercial mixture containing also gramicidins A, B and C, by E. R. Squibb and Sons Ltd., Moreton, Cheshire. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and carbonyl cyanide *p*-chlorophenylhydrazone were gifts from Dr P. G. Heytler.

RESULTS

The antibiotics may be classified into three groups depending on their ability to induce H^+ permeability in membranes. Group I (valinomycin, the actins and enniatin) does not affect H^+ permeability, group II (nigericin, dianemycin, monensin and compounds X-464, X-537A and X-206) induces free H^+ movement, and group III (gramicidin) induces only a limited H^+ permeability.

Group I

Valinomycin. Smectic mesophases were formed in 100mM-potassium mucate (hydroxyadipate) buffered with 3mM-tris phosphate, pH7·0. The external medium was exchanged for 145mM-choline

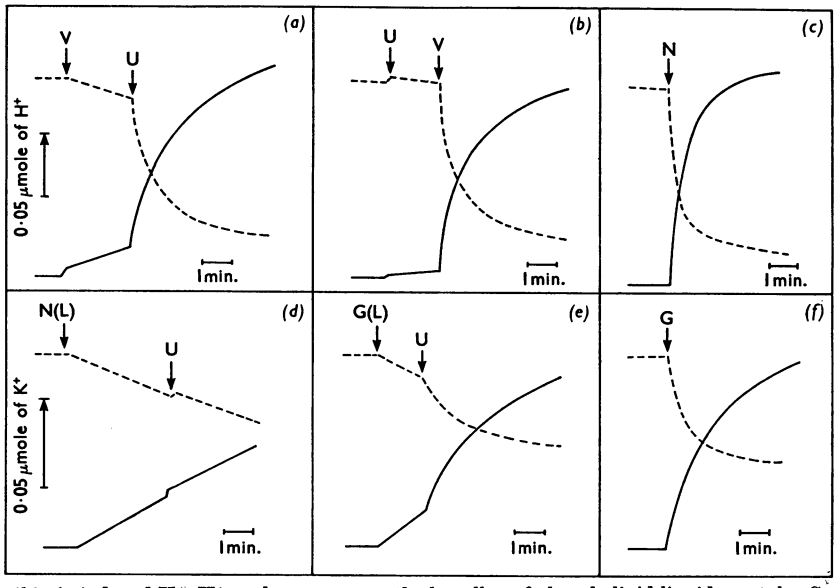


Fig. 1. Antibiotic-induced K^+–H^+ exchanges across the lamellae of phospholipid liquid crystals. Smectic mesophases of lecithin–10% dicetyl hydrogen phosphate containing 100mM-potassium mucate were prepared as described in the text, and external medium was exchanged for 145mM-choline chloride–0·5mM-KCl–0·75mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH6·65, on a Sephadex column. A 0·5ml. sample of the dispersion emerging from the column was suspended in 6·5ml. of the same medium at 30°. The final lipid concentration was 0·23μmole of lipid phosphate/ml. Additions were: V, 0·5μg. of valinomycin; U, 0·26μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (final concn.); G, 0·5μg. of gramicidin D; N, 0·5μg. of nigericin; G(L), 0·005μg. of gramicidin D; N(L), 0·005μg. of nigericin. In each case the upper recording (----) indicates pH changes and the lower one (——) indicates changes in K^+ concentration. The response of the electrodes was linear with respect to concentration of K^+ and H^+ over the ranges of ΔH^+ and ΔK^+ used.

chloride, 0·75mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid and 0·5mM-potassium chloride, pH6·7, on a Sephadex column. The H^+ and K^+ concentrations in the external medium were followed continuously by using selective glass electrodes. The addition of valinomycin even at high concentrations produced only a slow K^+ efflux, but this was greatly stimulated by the addition of agents that uncouple electron transport from phosphorylation (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 2,4-dinitrophenol, carbonyl cyanide *p*-chlorophenylhydrazone, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole and 5-chloro-2-trifluoromethylbenzimidazole), when K^+ efflux was accompanied by H^+ influx (Fig. 1*a*). The uncoupling agent alone did not produce a significant rate of K^+ efflux (Fig. 1*b*).

Since neither choline nor mucate is a penetrant, K^+ cannot leave the lipid structures unless H^+ can enter in exchange. Movement of K^+ alone would set up a membrane potential, which would retard further K^+ movement. It may be inferred that valinomycin makes the membranes permeable to K^+ but not H^+, and that uncoupling agents render the membrane proton-permeable.

Isotope exchanges were studied as described in the Methods and Materials section. Table 1 shows that valinomycin produced rapid $^{42}K^+$–K^+, $^{86}Rb^+$–Cs^+ and $^{134}Cs^+$–Cs^+ exchange but extremely slow $^{22}Na^+$–Na^+ and $^{22}Na^+$–Li^+ exchange. In separate experiments it was shown that no volume change occurred on adding the antibiotic, and hence there was no net salt movement and the results are valid measures of isotope exchange.

Valinomycin induced a rapid $^{42}K^+$–K^+, $^{86}Rb^+$–K^+ (Fig. 2) and $^{134}Cs^+$–K^+ exchange in erythrocytes, as measured by isotope uptake. It did not induce $^{22}Na^+$–K^+ exchange. Chappell & Crofts (1966) found that valinomycin did not produce K^+ efflux from erythrocytes suspended in choline chloride; however, the addition of an uncoupling agent in the presence of valinomycin induced rapid K^+ efflux and H^+ uptake, as with smectic mesophases (Henderson & Chappell, 1967; see also Harris & Pressman, 1967).

It is inferred that valinomycin renders erythrocytes and artificial phospholipid membranes permeable to K^+, Rb^+ and Cs^+, but has very little effect on their permeability to Li^+, Na^+ or H^+.

Actin homologues. The addition of monactin or

Table 1. *Isotope exchange across smectic mesophase membranes*

The cation appearing outside a dialysis bag in a 10 min. period (see the Methods and Materials section) was converted into a percentage fraction of that present inside the bag at the beginning of the 10 min. Four values of these rate constants (% efflux/10 min.) were taken before antibiotic addition, and were less than 1% for all the exchange systems. Each value below is the mean ± S.E.M. of four values obtained after addition of each antibiotic to a smectic mesophase containing the cations indicated. The dialysis bags contained 2·3 μmoles of lipid phosphate/ml. Antibiotics were added at saturating concentrations: valinomycin, 0·5 μg./ml.; gramicidin, 1 μg./ml.; monactin and nonactin + monactin, 2·5 μg./ml.; enniatin A, 20 μg./ml. The temperature was 20°.

	Rate constants (% efflux/10 min.)				
Antibiotic	$^{134}Cs^+$–Cs^+	$^{86}Rb^+$–Cs^+	$^{42}K^+$–K^+	^{22}Na–Na^+	$^{22}Na^+$–Li^+
Valinomycin	45·9 ± 3·7	48·0 ± 2·2	50·6 ± 2·7	1·5*	1·3*
Actin mixture	41·8 ± 2·6	46·1 ± 3·0	45·4 ± 2·9	3·5 ± 0·5	1·7*
Monactin	43·2 ± 3·0	45·3 ± 4·2	47·1 ± 3·1	12·9 ± 0·8	2·0*
Enniatin A	47·5 ± 5·2	48·2 ± 2·3	52·8 ± 2·0	24·0 ± 1·0	13·7 ± 0·8
Gramicidin	46·9 ± 1·4	49·3 ± 3·4	56·9 ± 7·4	42·6 ± 0·8	38·2 ± 2·5

* Not significantly different from controls.

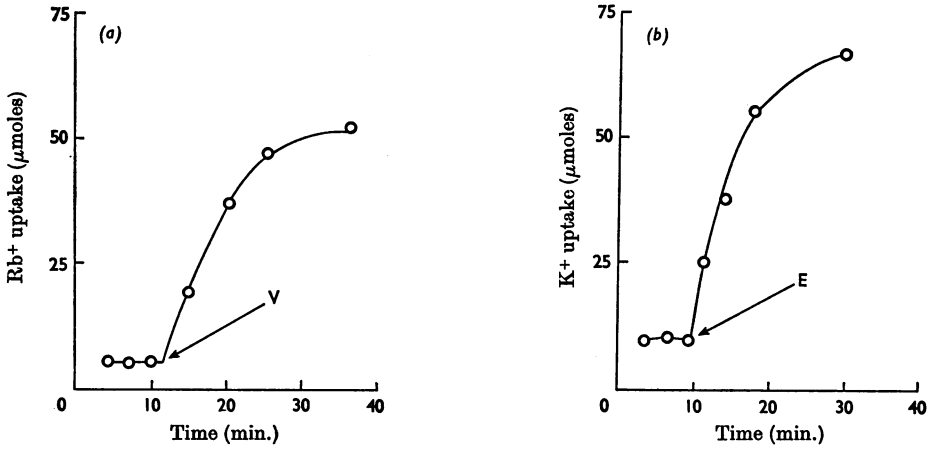


Fig. 2. Antibiotic-induced uptake of cations into erythrocytes. The suspension contained 0·75 ml. of washed erythrocytes in 7·6 ml. of 75 mM-KCl (or 75 mM-RbCl)–70 mM-choline chloride–6 mM-tris chloride, pH 7·30. The temperature was 30°. The medium was enriched with $^{42}K^+$ or $^{86}Rb^+$, and cation uptake into the erythrocytes was measured as described in the text. The additions were: V, 15 μg. of valinomycin; E, 100 μg. of enniatin A. A Jena combination pH electrode was utilized to monitor the pH of the medium simultaneously. No significant pH change occurred when the antibiotics were added (trace not shown).

nonactin to smectic mesophases containing potassium mucate produced only a very slow K^+ efflux; however, a rapid K^+–H^+ exchange was obtained on the further addition of a mitochondrial uncoupling agent. When the lipid was dispersed in sodium mucate, the actins, but not valinomycin, caused an Na^+–H^+ exchange in the presence of an uncoupling agent. Isotope-exchange experiments showed that both monactin and nonactin allowed a rapid $^{42}K^+$–K^+ and Rb^+–Cs^+ exchange. Monactin also gave a significant rate of $^{22}Na^+$–Na^+ exchange, but that induced by nonactin was very slow (Table 1).

It has already been reported that the actins released K^+ from erythrocytes suspended in choline chloride only in the presence of an uncoupling agent (Fig. 3*a*) (Chappell *et al.* 1968). When erythrocytes were suspended in [^{42}K]potassium chloride or [^{86}Rb]rubidium chloride, the actins induced $^{42}K^+$–K^+ or $^{86}Rb^+$–K^+ exchange across the membrane.

The actins thus appeared to allow movement of Cs^+, Rb^+ and K^+ across lipid membranes. Na^+

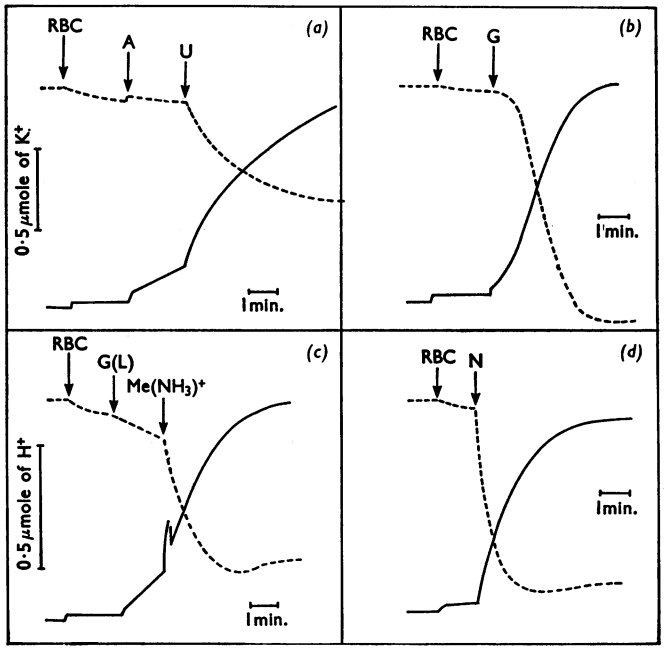


Fig. 3. K^+–H^+ exchange across erythrocyte membranes. A 25 μl. portion of washed erythrocytes was added to 4·95 ml. of 150 mM-choline chloride–3 mM-tris chloride, pH 7·30. The temperature was 30°. Additions were: RBC, erythrocytes; A, 20 μg. of nonactin–monactin mixture; G, 10 μg. of gramicidin D; G(L), 0·1 μg. of gramicidin D; N, 10 μg. of nigericin; U, 0·4 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (final concn.); $Me(NH_3)^+$, 1·0 mM-methylammonium chloride (final concn.). K^+ and H^+ movements are represented as in Fig. 1, and the electrode responses were linear with respect to concentration of H^+ or K^+.

movement was hindered; the relative effectiveness of the antibiotics in inducing Na^+ movement was: monactin > nonactin > valinomycin. Li^+ and H^+ movement were not observed with these compounds.

Enniatin A. In experiments similar to those described for valinomycin and the actins, it was found that enniatin A would not allow K^+–H^+ exchange across artificial phospholipid membranes in the absence of an uncoupling agent. However, enniatin A permitted relatively rapid exchanges between all the alkali-metal cations, as shown in Table 1. This antibiotic induced a greater permeability to K^+, Rb^+ and Cs^+ than to Na^+ or Li^+.

Enniatin promoted exchange of K^+ for K^+ (Fig. 2), Rb^+, Cs^+ or Na^+, but not H^+, across erythrocyte membranes. Thus enniatin A, although having a different cation specificity, was similar to valinomycin and the actins in that no proton permeability was observed in the absence of an uncoupling agent.

Considerably higher concentrations of this antibiotic were required to obtain rates of ion exchange comparable with those achieved with the actins and valinomycin. This is consistent with the observations by Mueller & Rudin (1967) on black lipid membranes and by Lardy *et al.* (1967) on mitochondria.

Group II

Group II contains nigericin, dianemycin, monensin and compounds X-206, X-537A and X-464. Owing to the limited supply of some of these, only nigericin and dianemycin were studied in detail on all the membrane systems.

Both antibiotics induced K^+ loss from potassium mucate-containing smectic mesophases suspended in choline chloride, and the cation efflux was accompanied by H^+ uptake (Fig. 1*c*); the H^+–K^+ exchange was not stimulated by uncoupling agents even when the concentration of antibiotic used limited the rate of cation movement (Fig. 1*d*). The H^+/K^+ ratio approached 1·0 if an impermeant anion, such as mucate, was sequestered inside the lipid structures, and cation–H^+ exchange still occurred if Rb^+ or Na^+ was the internal cation.

The ability of these compounds to induce rapid K^+–H^+ exchange across the erythrocyte membrane has already been reported (Henderson & Chappell, 1967; Pressman *et al.* 1967). Neither carbonyl

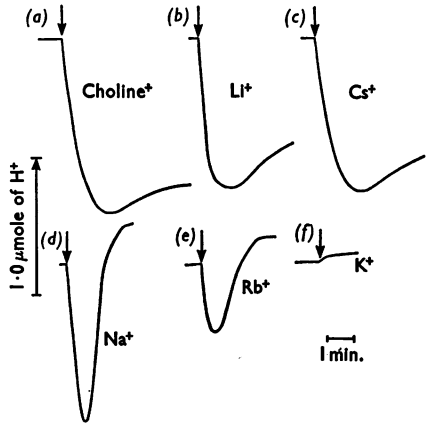


Fig. 4. Nigericin-induced pH changes across erythrocyte membranes. The conditions were those described in the legend to Fig. 3, but the 150mM-choline chloride was replaced successively by 150mM-LiCl, -CsCl, -NaCl, -RbCl and -KCl, all buffered with 3mM-tris chloride at pH7·3. The arrows indicate the point of addition of 10μg. of nigericin. A downward deflexion reflects alkalinization of the external medium.

cyanide *p* - trifluoromethoxyphenylhydrazone, methylamine nor NH_4^+ enhanced the rates of H^+ or K^+ movement. Fig. 4 shows the effect of substituting different cations for choline in the isoosmotic suspension medium. In lithium chloride and caesium chloride nigericin produced a straightforward influx of H^+ similar to that observed with choline, whereas with sodium chloride and rubidium chloride a reversal of the H^+ uptake occurred. By labelling the external cation with radioactive isotope, the shape of these pH changes was shown to be related to the rate of antibiotic-induced cation uptake. This experiment has been repeated for each of the group II antibiotics with radioactive isotopes of Cs^+, Na^+, K^+ and Rb^+, and it was found that each compound gave a unique set of pH changes for a series of experiments differing only in the type of alkali-metal cation present (Henderson, 1968).

Results for nigericin and monensin in Na^+- and Rb^+-containing media are presented in Fig. 5. At the cation concentrations used, nigericin produced a rapidly reversing alkaline pH shift in both rubidium chloride and sodium chloride (increasing the con-

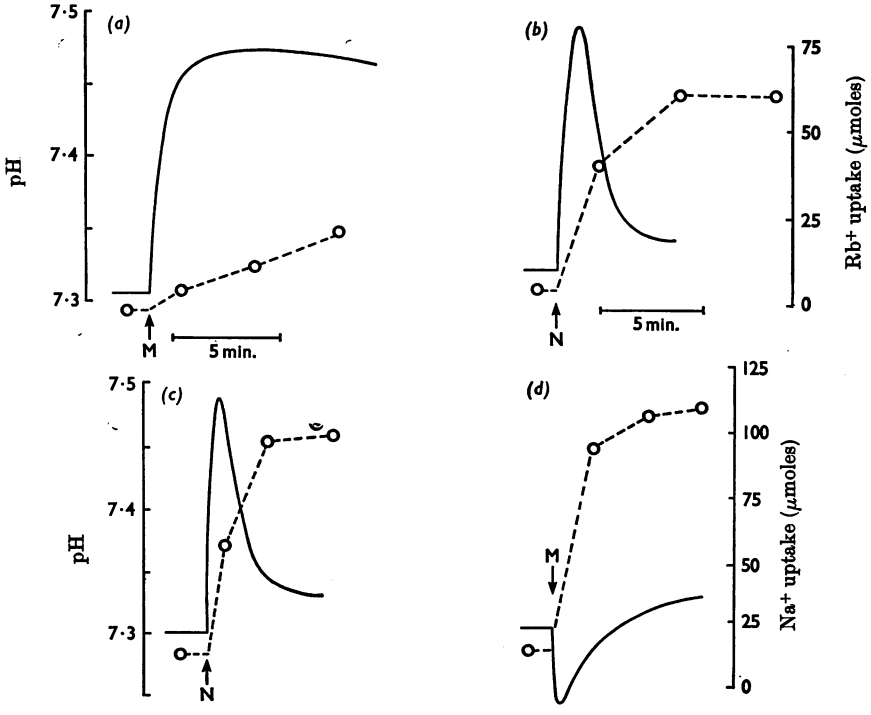
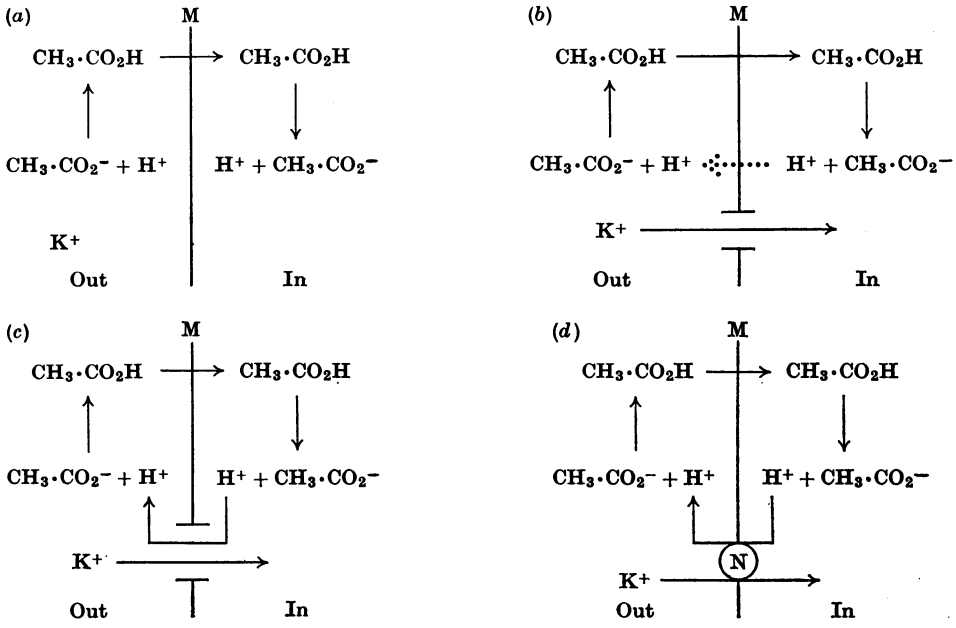


Fig. 5. H^+–cation exchanges across erythrocyte membranes. For the two upper recordings (*a* and *b*) 0·75ml. of washed erythrocytes was suspended in 7·6ml. of a medium containing 77·5mM-RbCl (enriched with $^{86}Rb^+$)–70mM-choline chloride–16mM-tris chloride, pH7·3. For the two lower recordings (*c* and *d*) 0·75ml. of erythrocytes was suspended in 7·6ml. of 150mM-NaCl–16mM-tris chloride, pH7·4. Uptake of $^{86}Rb^+$ or $^{22}Na^+$ into the erythrocytes (○--○) was measured by the sampling technique described in the text, and the pH of the medium (——) was recorded with a Jena combination glass electrode. Additions were: M, 300μg. of monensin; N, 30μg. of nigericin. The temperature was 30°.



Scheme 1. Mechanism for the entry of acetate into mitochondria. (*a*) No antibiotic added: no swelling. (*b*) Valinomycin or gramicidin D added: slow swelling enhanced by uncoupling agents. (*c*) More rapid swelling at high concentrations of gramicidin D. (*d*) Very rapid swelling in the presence of nigericin.

centration of Rb^+ progressively decreased the size of the pH cycle) and the reversal coincided with Rb^+ or Na^+ uptake. By contrast, in Na^+-containing medium monensin induced an acid–alkaline cycle, although the rate of Na^+ uptake was similar to that induced by nigericin, and in rubidium chloride monensin gave a straightforward H^+ influx, unaccompanied by $^{86}Rb^+$. In most of these experiments it was not possible to measure the simultaneous efflux of K^+ from the erythrocytes, but in all cases those concentrations of antibiotic were used that produce a rapid K^+ loss in media containing only choline as cation.

These examples demonstrate two principles derived from a number of such experiments: first, that the slower the uptake of external cation, the greater is the extent and irreversibility of the alkaline pH change; secondly, that the direction of the pH change reflects the preferential selectivity of antibiotic for internal or external cation. Thus monensin gives an initial acid change in sodium chloride medium because it has a greater selectivity for the external Na^+ over internal K^+. Like monensin, dianemycin gave an acid pH change in sodium chloride medium, whereas compounds X-464 and X-537A initiated an alkaline–acid cycle similar to that shown for nigericin.

These experiments also showed that individual members of this class of antibiotics possess different cation selectivities (summarized in Table 3), and this has been confirmed by experiments on smectic mesophases that are described below.

Acetate crosses the mitochondrial membrane rapidly, although Cl^-, a much smaller anion, is unable to do so. This has been explained (Chappell & Crofts, 1966) by assuming that a biological membrane imposes little restraint on the passage of small neutral molecules: in this case, undissociated acetic acid (Scheme 1*a*), which dissociates to an acetate anion and an H^+ ion after crossing the membrane. Thus both mitochondria (Chappell & Crofts, 1966) and phospholipid liquid crystals (Bangham *et al.* 1967) swell very rapidly in ammonium acetate.

When smectic mesophases or non-respiring mitochondria were suspended in potassium acetate, no swelling occurred until valinomycin, actin or gramicidin was added to induce K^+ permeability. With all three compounds, subsequent addition of uncoupling agent markedly increased the rate of swelling (Fig. 6). The uncoupling agent facilitates the entry of K^+ by allowing H^+ to leave the mitochondria, also discharging the pH differential, which becomes limiting on the entry of acetate (Scheme 1*b*). With nigericin and dianemycin, however, the swelling was very rapid (Fig. 6) and was not enhanced by uncoupling agents. It is inferred that these compounds facilitate H^+

transfer across the mitochondrial membrane much more readily than the group I antibiotics (Scheme 1*d*).

This interpretation was confirmed by measuring the K^+ efflux from mitochondria suspended in iso-osmotic buffered choline chloride, with respiration inhibited by rotenone and antimycin. Under these conditions valinomycin at any concentration only induced K^+ loss when an uncoupling agent was present to allow K^+–H^+ exchange (see also Carafoli & Rossi, 1967), whereas dianemycin (Fig. 7) or nigericin allowed immediate K^+ loss and H^+ uptake. The re-equilibration of the pH change observed in these experiments is believed to be due to a secondary efflux of anions from the mitochondria, presumably exchanging for external OH^-.

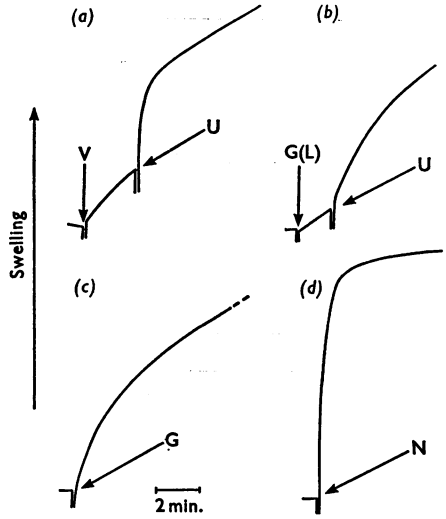


Fig. 6. Swelling of mitochondria in potassium acetate. Mitochondria (approx. 7 mg. of protein) were suspended in 2·4 ml. of 145 mM-potassium acetate–5 mM-tris chloride, pH 7·4, containing 0·2 μg. of antimycin A and 0·2 μg. of rotenone. Additions were: V, 5 μg. of valinomycin; U, 0·4 μM-carbonyl cyanide *p*-chlorophenylhydrazone (final concn.); G(L), 0·5 μg. of gramicidin D; G, 5 μg. of gramicidin D; N, 0·5 μg. of nigericin. Swelling was monitored by measuring the scattering of 550–600 mμ light at an angle of 45° to the incident beam.

Nigericin and dianemycin tended to reverse the changes induced in respiring mitochondria by the group I antibiotics (Graven *et al.* 1966*a*); inhibition of respiration on addition of nigericin occurred with all substrates except succinate, β-hydroxybutyrate and proline (Lardy *et al.* 1967). Several workers have described the inhibition of valinomycin- or actin-accelerated respiration by uncoupling agents (Graven *et al.* 1967; Harris *et al.* 1967*a*), and Chappell & Crofts (1965) showed that NH_4^+ had the same effect on gramicidin-induced succinate respiration in low-K^+ media. These results have been extended to show that the same pattern of respiratory inhibition occurred whether an uncoupler, nigericin or NH_4^+ was the active agent (Table 2).

Since each of these agents has the ability to carry H^+ across membranes, it is suggested that it is this property that leads to the K^+ movement and shrinkage which occurs whatever the substrate present. The reason for the substrate specificity is discussed in more detail below.

Group III

Gramicidin. The ability of gramicidin to induce K^+–H^+ or K^+–Na^+ exchange across the erythrocyte

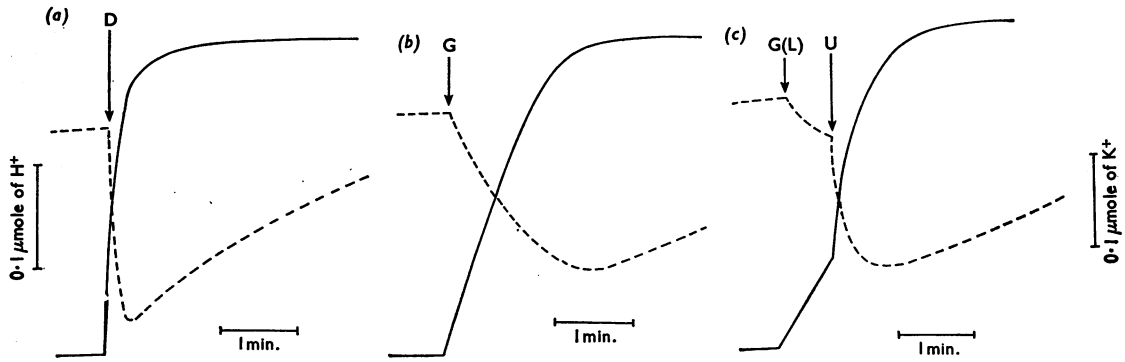


Fig. 7. K^+–H^+ exchanges across the mitochondrial membrane. Mitochondria (7·4 mg. of protein) were suspended in 6·9 ml. of 145 mM-choline chloride–0·43 mM-KCl–2 mM-tris chloride, pH 7·4, containing 0·2 μg. of antimycin A and 0·4 μg. of rotenone. Additions were: D, 0·5 μg. of dianemycin; G, 10 μg. of gramicidin D; G(L), 0·5 μg. of gramicidin D; U, 0·14 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (final concn.). Upper recording (-----), H^+ electrode; lower recording (——), K^+ electrode. The temperature was 30°.

Table 2. *Effects of* H^+*-carrying agents on antibiotic-accelerated respiration*

Mitochondria were incubated in 4ml. of medium containing 130mM-choline chloride, 0·6mM-tris phosphate, 5mM-tris chloride, 10mM-KCl (8mM-KCl for the valinomycin experiments) and substrates as indicated. A 0·5μg. portion of gramicidin or valinomycin was added and, when respiration had reached a steady rate, either 3·75mM-NH_4Cl or 0·5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CCFP). Tris salts of all substrates except for sodium β-hydroxybutyrate were used. The pH was 7·4 and the temperature was 30°. (The solubility of oxygen was 0·445μg.atom of O/ml.) Oxygen concentration was measured with a Clark-type oxygen electrode, and the rates of oxygen uptake are expressed as μg.atoms of O/min./g. of protein.

Substrate	Gramicidin*	Gramicidin + NH_4^+*	Valinomycin†	Valinomycin + CCFP†
3mM-Glutamate	176	23	158	15
3mM-Succinate (+ rotenone)	175	142	147	181
3mM-Citrate	95	14	64	10
6mM-β-Hydroxybutyrate	88	74	66	70

* 1·45mg. of mitochondrial protein/ml.
† 1·22mg. of mitochondrial protein/ml.

membrane has already been reported (Chappell & Crofts, 1966).

Table 1 shows that gramicidin restricted Li^+ and Na^+ movement when compared with Cs^+, Rb^+ or K^+, but the rates were still considerably greater than those induced by the group I antibiotics on artificial phospholipid membranes.

With erythrocytes the gramicidin-induced pH change accompanying K^+ extrusion disappeared if iso-osmotic choline chloride was replaced by any of the alkali-metal chlorides, and enrichment of these media with radioactive Cs^+, Rb^+, K^+ or Na^+ showed that cation uptake was replacing the H^+ influx. This is in contrast with the observations with group II compounds, where a transient pH change nearly always accompanied net cation–cation exchange (Henderson, 1968).

The ability of gramicidin to catalyse H^+ transfer depended on its concentration. Up to approx. 1μg./ml., uncoupling agents, ammonia or methylamine (Fig. 2*c*) considerably enhanced the rates of H^+ uptake and K^+ efflux. The same phenomenon was observed for K^+–H^+ exchange in smectic mesophases (Fig. 1*e*) or mitochondria (Fig. 7) or for gramicidin-induced swelling of mitochondria in potassium acetate (Fig. 6 and Scheme 1*c*).

The preference of gramicidin for cation–cation rather than cation–H^+ exchange was also indicated by the following experiment. For erythrocytes suspended in choline chloride a lag was observed before the rates of K^+–H^+ movement became maximal, even at saturating gramicidin concentrations. The medium for these experiments contained 1mM-potassium chloride, necessary for optimum operation of the K^+-sensitive electrode, and when this was labelled with $^{42}K^+$ it was found that a very rapid K^+ uptake occurred during the lag phase, although the K^+ electrode indicated a small net efflux from the erythrocytes. The influx of $^{42}K^+$ reversed as the H^+ uptake began to increase towards the maximal rate. Presumably a very rapid initial K^+ influx kept pace with a rapid K^+ efflux, but then equilibration of the prevailing concentration gradient necessitated a net K^+–H^+ movement. No such $^{42}K^+$ uptake appeared with nigericin, which did not show a lag before K^+–H^+ exchange achieved a maximal rate.

Gramicidin induced swelling of rotenone + antimycin-treated mitochondria suspended in potassium acetate, and the rate of volume increase was enhanced by uncoupling agents (Fig. 6) or NH_4^+. With respiring mitochondria, this concentration of gramicidin (0·2μg./ml.) would have produced maximal cation uptake, swelling and accelerated respiration. In the potassium acetate system, increasing the gramicidin concentration above this level stimulated the rate of swelling (Fig. 6) towards that obtained with 'normal' low concentrations of nigericin or dianemycin. Fig. 7 shows that a higher concentration of gramicidin also permitted more rapid K^+–H^+ exchange across the mitochondrial membrane.

To summarize, these experiments indicated that gramicidin catalyses K^+ transfer more readily than H^+ transfer. This is in contrast with nigericin and dianemycin, which facilitated similar rates of K^+ or H^+ movement.

Confirmation of cation selectivities

Smectic mesophases of lecithin–10% dicetyl hydrogen phosphate containing 40mM-potassium chloride were suspended in iso-osmotic solutions of the other alkali-metal chlorides. All solutions were buffered at pH 7·0 with 5mM-tris phosphate. Various antibiotics were added, and changes in E_{450} of the suspensions were followed.

The addition of nigericin to this system would

allow either K^+–cation or K^+–H^+ exchange. K^+–H^+ exchange would be followed by Cl^-–OH^- exchange and the net result is then loss of K^+ and Cl^- with consequent shrinking of the lipid structures; K^+–cation exchange, however, would not be followed by Cl^-–OH^- exchange and then no shrinking would occur. The rate and extent of shrinking in the various alkali-metal chlorides therefore depends on the relative rates of K^+–H^+ and K^+–cation exchanges induced by a particular antibiotic. This argument applies to all group II antibiotics and to gramicidin.

With antibiotics that did not permit H^+ transfer, the presence of uncoupling agents is necessary to allow possible K^+–H^+ exchange. Then, as before, if an antibiotic induces a rapid K^+–cation exchange, this is not followed by Cl^-–OH^- exchange and no shrinking results. If an antibiotic allows only a slow K^+–cation exchange, however, K^+–H^+ exchange is preferred in the presence of an uncoupling agent; this is followed by Cl^-–OH^- exchange, net loss of potassium chloride and shrinking. Thus the rate and extent of shrinking depends on the ease of K^+–cation exchange mediated by an antibiotic. Further evidence for this interpretation is presented elsewhere (McGivan, 1968).

Fig. 8 shows that valinomycin, enniatin A and the actins caused volume changes (in the presence of an uncoupling agent) that were consistent with the ion selectivities deduced from the isotope-exchange experiments. With nigericin the extent of the

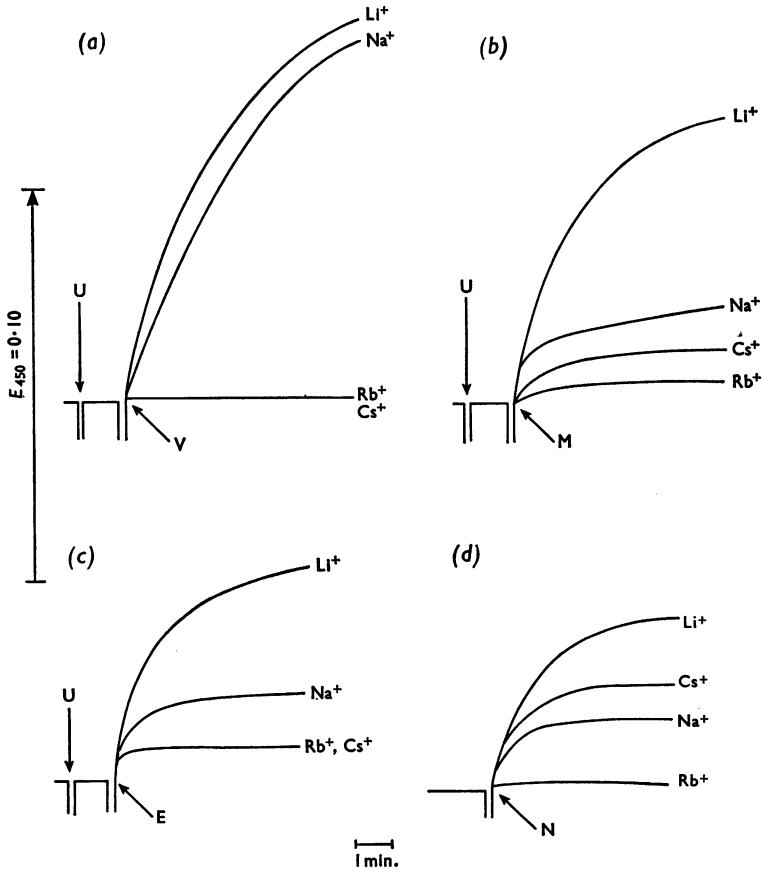


Fig. 8. Antibiotic-induced volume changes of phospholipid liquid crystals. A 90% lecithin–10% dicetyl hydrogen phosphate mixture was dispersed in 40mM-KCl–5mM-tris phosphate, pH 7·0 (15μmoles of lipid phosphate/ml.). A 40μl. sample of the dispersion was added to 2·45ml. of 40mM-LiCl, -NaCl, -RbCl or -CsCl buffered at pH 7·0 with 5mM-tris phosphate. The initial E_{450} was 0·88–0·95. Additions: U, 0·2μM-carbonyl cyanide *p*-chlorophenylhydrazone (final concn.); V, 0·5μg. of valinomycin; M, 2·5μg. of monactin; E, 20μg. of enniatin A; N, 2·5μg. of nigericin. The temperature was 30°.

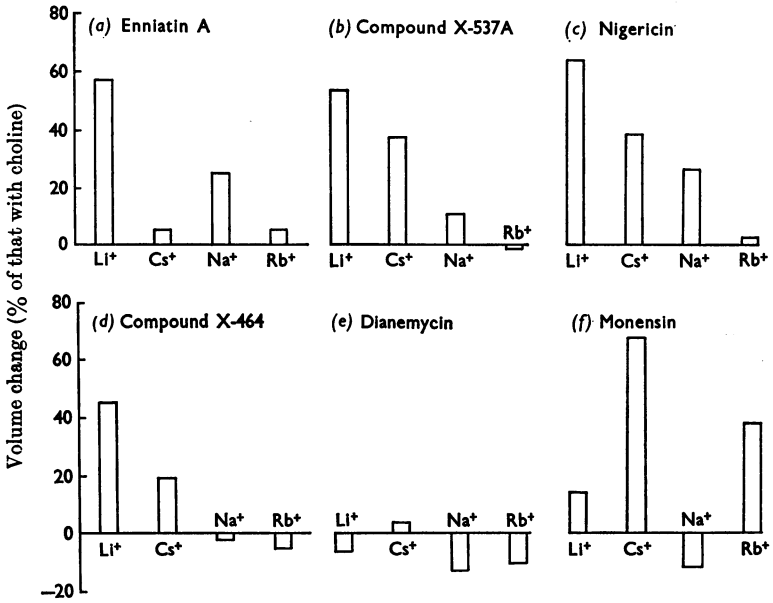


Fig. 9. Antibiotic-induced volume changes of phospholipid liquid crystals. Dispersions containing 40 mM-KCl–1 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 6·55, were suspended in 2·4 ml. of 40 mM-alkali-metal chlorides buffered at pH 6·55 with 1 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid. The initial E_{450} of the suspensions was 0·55–0·80. The E_{450} induced by 0·5 μg. of each of the nigericin-type antibiotics and by 20 μg. of enniatin A (in the presence of 0·2 μM-carbonyl cyanide *p*-chlorophenylhydrazone) was recorded. The results are presented as a percentage of E_{450} in choline chloride as described in the text. The temperature was 25°.

Table 3. *Selectivity of antibiotics for alkali-metal cations and* H^+

The selectivities were deduced from the experiments on erythrocytes and smectic mesophases described in the text.

Antibiotics	Cation selectivity	H^+ permeability
Valinomycin	K^+, Rb^+, $Cs^+ \gg NH_4^+$, Na^+, $MeNH_3^+$, Li^+	None
Monactin	K^+, Rb^+, Cs^+, NH_4^+, $Na^+ \gg MeNH_3^+$, Li^+	None
Nonactin + monactin	K^+, Rb^+, $Cs^+ > NH_4^+$, $Na^+ \gg MeNH_3^+$, Li^+	None
Enniatin A	K^+, Rb^+, $Cs^+ > Na^+ > Li^+$	None
Gramicidin D	K^+, Rb^+, Cs^+, NH_4^+, Na^+, Li^+, $MeNH_3^+$	Limited: further addition of uncoupling agents stimulates at low gramicidin concentrations
Nigericin	K^+, $Rb^+ > Na^+ > Cs^+ > Li^+$	All induce H^+ permeation: further addition of uncoupling agents does not enhance H^+ movement, even at low antibiotic concentrations
Dianemycin	Na^+, K^+, Rb^+, Li^+, Cs^+	
Monensin	$Na^+ > K^+$, $Li^+ > Rb^+ > Cs^+$	
Compounds X-464,* X-537A and X-206†	K^+, $Rb^+ > Na^+ > Cs^+ > Li^+$	

* Compound X-464 may be identical with nigericin (Lardy *et al.* 1967).
† Preliminary results only.

volume changes indicated that this antibiotic facilitated movement of cations in the order: K^+, $Rb^+ > Na^+ > Cs^+ > Li^+ >$ choline$^+$.

The exact extent of the volume changes varied from one lipid dispersion to another, and all comparisons were made with one preparation. With the group II compounds it was also a function of the concentration of antibiotic added: the higher the concentration, the less the apparent discrimination between different cations. To facilitate the comparison between different antibiotics the same concentration of each was used, and the total

volume changes for each cationic series were expressed as a percentage of that obtained in choline chloride. The results are presented as a series of histograms in Fig. 9. Nigericin and compounds X-464, X-206 and X-537A gave similar patterns of specificity. Dianemycin exhibited little discrimination between any of the alkali-metal cations, and monensin showed a preference for Na^+, K^+ and Li^+ when compared with Cs^+ or Rb^+. The cation selectivities of the group II compounds deduced by this method are therefore consistent with those indicated by pH changes and isotope exchanges across erythrocyte membranes. They are summarized in Table 3.

DISCUSSION

Observations of the antibiotic-induced passive cation exchanges across erythrocyte or artificial phospholipid membranes show similar cation selectivities (Table 3) to those previously described for respiring mitochondria (for review see Lardy *et al.* 1967). NH_4^+ substituted for an uncoupling agent in enhancing the K^+–H^+ exchange induced by gramicidin, monactin or nonactin, but not that induced by valinomycin. Since it is necessary for NH_4^+ to be transported by the antibiotic to facilitate H^+ transfer (Chappell & Crofts, 1965, 1966), it has been inferred that valinomycin does not transport NH_4^+ (Table 3). Similar experiments showed that methylammonium ions were transported by gramicidin but not by the actins or valinomycin (Henderson, 1968). The inability of the group I class of compounds to induce proton permeability can be demonstrated with non-respiring mitochondria by using the potassium acetate swelling system or by measurements of K^+–H^+ exchange (see also Carafoli & Rossi, 1967). Thus the selectivity of each antibiotic towards H^+ is the same on each of the three membrane systems.

The mechanism by which an antibiotic catalyses cation movement across the membrane may be considered in two ways. First, J. B. Chappell (see Pressman, 1965) has suggested that valinomycin and gramicidin create pores in membranes, through which cations with a small enough hydration shell could pass. The order of hydrated ion size for the alkali-metal cations is: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Stern & Amis, 1959). Thus a cyclic configuration (Sarges & Witkop, 1965) of gramicidin would allow all these cations, and also H^+, to traverse the membrane, whereas the smaller cyclic molecules of the actin series and valinomycin show a selectivity towards the smaller cations, and, as shown above, exclude H^+. If all the antibiotics formed complexes with fully hydrated cation species, then enniatin A, which has six acid residues compared with 12 acid residues in valinomycin (see review by Lardy *et al.* 1967), should exhibit greater selectivity. However, the results above indicate that enniatin A is less specific than valinomycin (see Table 1) in that it allowed transport of Na^+ and Li^+ as well as of Cs^+, Rb^+ and K^+.

All of the group II antibiotics exhibited a greater selectivity for Na^+, K^+ or Rb^+ than Cs^+ on both the erythrocyte and phospholipid membranes, despite the small hydrated radius of Cs^+, and two were more selective for Na^+ than K^+. Monensin, the only one of these compounds whose structure has been completely elucidated (Agtarap, Chamberlain, Pinkerton & Steinrauf, 1967), tended to exclude both Cs^+ and Rb^+ in favour of Na^+, K^+ or Li^+, in complete opposition to the selectivity of valinomycin. A similar specificity has been reported for the action of monensin on mitochondria (Estrada-O, Rightmire & Lardy, 1967*c*). Data on the degree of hydration of H^+ in water are not available, but its very small crystal radius would presumably qualify it for an hydration shell at least as large as that of solvated Li^+. This is entirely compatible with its exclusion by valinomycin and actin, but not by gramicidin. With the group II compounds, however, where induced H^+ movement is as fast as that of K^+, leading to the transient pH changes observed even when a net cation–cation exchange occurred, presumably the H^+ permeation is by another mechanism, the obvious candidate being combination with the carboxyl group. The above results therefore demonstrate that the antibiotic–cation complex does not necessarily involve fully hydrated cations. Molecular models of several such types of co-ordination complexes have been discussed by Mueller & Rudin (1967) and by Lardy *et al.* (1967), but the model approach has its limitations: their proposal for the conformation of the actin–cation complex is unlike that deduced by X-ray-diffraction studies (Kilbourn, Dunitz, Pioda & Simon, 1967).

A second manner in which the antibiotics could transfer cations across membranes has been suggested by Pressman *et al.* (1967). Rather than a static pore the antibiotics would form a lipid-soluble complex with the cation, and this passes across a membrane to release the cation on the other side. X-ray-diffraction studies of monensin (Agtarap *et al.* 1967) and nonactin (Kilbourn *et al.* 1967) complexes with cations have shown the cation to be completely surrounded by a hydrophobic 'shell' of antibiotic molecule, in accordance with this proposal.

Pressman *et al.* (1967) have shown that valinomycin, dianemycin and nigericin all carried isotopically labelled Rb^+ from an aqueous into an organic phase. With valinomycin, but not with nigericin or dianemycin, this process was enhanced by the presence of the lipophilic laurate anion. It was inferred that the nigericin and dianemycin

complexes with cations were uncharged, whereas those with valinomycin carried a positive charge, a conclusion supported by the failure of nigericin to change the electrical resistance of black lipid membranes.

One corollary of this failure to decrease the membrane resistance is that the nigericin antibiotics are lipid-soluble only when their carboxylic acid group is neutralized by either an H^+ ion or a cation (see also Jackson, Crofts & Stedingk, 1968). The valinomycin group of antibiotics must be lipid-soluble both as the free molecules and as charged complexes.

A second corollary is that the cation–cation exchange induced by a nigericin-type compound will be coupled in a 1:1 ratio. Glynn (1967) has pointed out that, if this is so, K^+ loss from non-respiring mitochondria promoted by nigericin could not induce a charge separation, although that induced by valinomycin could. The converse would also apply, namely that the movement of cations induced by valinomycin could discharge a pre-existing membrane potential, whereas that by nigericin could not.

The chemiosmotic theory of oxidative phosphorylation (Mitchell, 1966) suggests that electron transport maintains an electrical potential difference (positive outside) across the cristal membrane of respiring mitochondria. Mitchell (1966) has suggested that uncoupling agents act by moving H^+ down the potential gradient, so dissipating the energy required for ATP synthesis, whereas valinomycin and gramicidin have the same effect through movement of K^+. However, it has been shown that only gramicidin, not valinomycin or actin, uncouples phosphorylation from mitochondrial electron transport (Höfer & Pressman, 1966; Harris *et al.* 1967*a*). It is proposed here that the acceleration of the proton pump after the discharge of the potential by valinomycin-induced K^+ movement replaces the potential with a pH gradient, which may still be used to drive ATP synthesis (Mitchell, 1966; cf. Glynn, 1967). Since gramicidin allows both H^+ and K^+ to cross the membrane, it would collapse both the potential and pH differences, so completely uncoupling phosphorylation from respiration. A better illustration of this point is provided by the experiments of Stedingk & Baltscheffsky (1966) with chromatophores of *Rhodospirillum rubrum*. The light-induced high-energy state of chromatophores was found to be associated with a pH difference across the membrane; this was enhanced by valinomycin, but gramicidin led to its dissipation. Jackson *et al.* (1968) have shown that the collapse of the pH gradient by gramicidin is accompanied by loss of light-induced ATP synthesis, although photophosphorylation is preserved in the presence of valinomycin.

Nigericin would also allow H^+ to cross the membrane but, as explained above, another cation would have to be carried in the opposite direction for the H^+ movement to continue. There would then be no effect on a membrane potential and uncoupling should not be observed.

In response to a membrane potential (assumed to be negative inside the mitochondrion) the charge-transferring valinomycin antibiotics allow K^+ movement inwards down the electric potential gradient and up the concentration gradient. Nigericin, however, only permits K^+ to move down the concentration gradient since its complex with K^+ is uncharged.

Cation movements induced by the antibiotics might be expected to lead to secondary anion movements if the latter were coupled to OH^- antiport, as suggested by Chappell & Crofts (1966) and Chappell & Haarhoff (1967), or H^+ symport, as described above for the acetate system (cf. Mitchell, 1967). Thus K^+ entry promoted by valinomycin or the actins gives rise to a transmembrane pH gradient (alkaline inside) due to acceleration of the proton pump, as discussed. Such a pH gradient would facilitate substrate anion accumulation and the rate of respiration (as found by Höfer & Pressman, 1966; Harris *et al.* 1967*a*). No pH gradient would be maintained in the presence of gramicidin, thus explaining its failure to increase respiration rates to the same extent as valinomycin or the actins (Harris *et al.* 1967*a*). On the other hand, the nigericin-induced K^+ efflux is coupled to an uptake of H^+, and the resulting internal acidity would be expected to facilitate substrate anion efflux in exchange for external OH^-. If the intramitochondrial substrate concentration became sufficiently low, respiration would be inhibited. Lardy *et al.* (1958) and Graven *et al.* (1966*a*) have observed that nigericin causes respiratory inhibition, which is relieved by increasing the external K^+ or substrate concentrations in accordance with the above scheme.

However, the substrate specificity of the inhibitions requires further consideration. As shown above, the specificity is preserved whether nigericin, valinomycin + uncoupling agent or gramicidin + NH_4^+ initiate the inhibitions (Table 2). The induction of membrane permeability to both K^+ and H^+ allows K^+ to leave the mitochondria down the concentration gradient; besides substrate anion, internal phosphate could accompany the K^+, and provision of this anion in the external medium was found to alleviate the respiratory inhibitions (Estrada-O *et al.* 1967*a*). In rat liver mitochondria the oxidation of glutamate via the phosphate-requiring oxidation of 2-oxoglutarate and the entry of malate, oxoglutarate, citrate and isocitrate are all dependent on an initial supply of phosphate (for review see Chappell, 1968), whereas succinate,

β-hydroxybutyrate or proline oxidations are independent of added phosphate. Hence loss of internal phosphate could be responsible for the observed substrate specificity of the inhibitions.

An alternative explanation for the action of both classes of antibiotics on mitochondria is provided by postulating the existence of a cation 'pump' in the membrane (Pressman, 1965; Höfer & Pressman, 1966; Pressman *et al.* 1967). By utilizing energy generated from respiration this would move cations into the mitochondrion against the concentration gradient, thereby facilitating the entry of anionic substrates. It has been proposed that valinomycin specifically stimulates this pump, and enhanced K^+ uptake stimulates a postulated K^+-dependent step in the synthesis of ATP from high-energy intermediates, so allowing phosphorylation to accelerate at a corresponding rate to electron transfer. In one interpretation both valinomycin and nigericin interact with the pump mechanism (Lardy *et al.* 1967), the former activating, the latter inhibiting, whereas in another nigericin differs by acting at 'random loci' in the membrane (Pressman *et al.* 1967). No explanation was offered as to why gramicidin, which stimulates K^+ uptake to the same extent as valinomycin, did not also accelerate phosphorylation of ADP, although it was recognized that induction of proton permeability by gramicidin might be the determining factor (Harris *et al.* 1967*a*). In none of our experiments was it necessary to invoke a specific cation pump to explain the antibiotic-induced exchanges.

At high concentrations nigericin, but not dianemycin, induces mitochondrial adenosine triphosphatase (Estrada-O *et al.* 1967*a*), and a brief report has appeared describing its ability to accelerate respiration (Estrada-O *et al.* 1967*b*). K^+ is an essential component for maximal effects, although some activity is observed with the other alkali-metal cations; in this Laboratory the order of efficiency in potentiating the respiratory acceleration was shown to be: $K^+ > Rb^+ > Na^+$, with very little activity in Cs^+ and Li^+, i.e. identical with the cation selectivities deduced from model systems (Table 3). An explanation of these effects could be that at high concentrations the negatively charged antibiotic becomes sufficiently soluble in the lipid membrane for it to act as a one-way charge carrier; movement of alkali-metal cations or H^+ could then discharge a membrane potential. Data for the partition coefficients of the nigericin anion between organic and aqueous phases are not at present available, but would provide some experimental evidence for this interpretation.

The effects of these antibiotics on passive and light-induced ion fluxes in *R. rubrum* chromatophores (Jackson *et al.* 1968) have also been interpreted in terms of the chemiosmotic hypothesis. The results are entirely compatible with this discussion of the mitochondrial system if it is assumed that the polarity of the potential is opposite to that in the mitochondria.

To summarize, it is suggested that the passive ion permeabilities induced by antibiotics in both mitochondrial and model membranes are identical. The unique effects of each class of antibiotics on mitochondrial respiration could be explained in these terms if a membrane potential were present, as suggested by the chemiosmotic hypothesis, and this renders the postulation of an inwardly directed cation pump unnecessary.

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