Gramicidin and Ion Transport in Isolated Liver Mitochondria

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1. Gramicidin caused H^+ production by liver mitochondria; this process was dependent on the presence of Na^+ , Li^+ , Rb^+ or Cs^+ . In the presence of one of these alkali-metal ions and phosphate, gramicidin caused mitochondrial swelling and increased oxygen consumption. 2. Uncoupling agents, anaerobic conditions or respiratory inhibitors both inhibited and reversed H^+ production and swelling. Both these processes could be supported by coupled electron transport through even a restricted portion of the respiratory chain. 3. NH_4^+ also caused stimulation of respiration in the presence of gramicidin. In this case phosphate was not required. NH_4^+ , in the presence of gramicidin, caused contraction of the mitochondria and a reversal of K^+ -induced swelling and H^+ production. 4. Uncoupling agents or NH_4^+ together with gramicidin caused the release of Sr^{2+} that had been accumulated by mitochondria in the presence of phosphate. 5. These results are discussed in relation to a postulated respiration-dependent H^+ pump located in the mitochondrial membrane. It is suggested that gramicidin allows alkali-metal ions to pass through the mitochondrial membrane.

Gramicidin has been used extensively as an uncoupling agent. Neubert & Lehninger (1962) showed that even at low concentrations gramicidin caused extensive swelling when mitochondria were suspended in buffered iso-osmotic potassium chloride solutions, but not when potassium chloride was replaced by sucrose. Pressman (1963) and Moore & Pressman (1964), using the antibiotic valinomycin, which in many respects has a close structural similarity to gramicidin, have shown that the action of this uncoupling agent is dependent on the presence of K^+ , Rb^+ or Cs^+ and of phosphate. Valinomycin causes the appearance of H^+ in the suspending medium and this effect is K^+ - but not phosphate-dependent. Pressman (1963) has indicated briefly that valinomycin and gramicidin have a similar action.

The present paper gives experimental evidence showing that gramicidin is indeed very similar in action to valinomycin in that it caused the release of H^+ from mitochondria in the presence of K^+ and an increase in rate of respiration in the presence of K^+ and phosphate. However, whereas valinomycin will act with K^+ and not with Na^+ or Li^+ (Moore & Pressman, 1964), gramicidin will act with all the alkali-metal ions tested and with NH_4^+ . It was discovered that gramicidin caused marked swelling of liver mitochondria suspended in the presence of K^+ and phosphate. This swelling was reversed

spontaneously when the mitochondrial suspension became anaerobic, or on the addition of respiratory inhibitors, uncoupling agents or NH_4^+ . At high K^+ and low sucrose concentrations gramicidin induced a swelling that was not reversed under these conditions. The relationship of these effects to the respiration-dependent accumulation of bivalent cations in the presence of phosphate, especially the accumulation of Sr^{2+} (Chappell, Cohn & Greville, 1963), has been investigated. It was deduced from measurements of changes in H^+ concentration that, under appropriate conditions, NH_4^+ in the presence of gramicidin caused the release of accumulated Sr^{2+} .

EXPERIMENTAL

The methods and most of the materials used in the present investigation were as described by Chappell & Crofts (1965a). Gramicidin was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and was a kind gift from Dr G. D. Greville. Strophanthin G was obtained from British Drug Houses Ltd., Poole, Dorset.

Considerable technical difficulty was experienced in recording small changes in pH in a medium containing little electrolyte. For this reason an iso-osmotic medium consisting of sucrose (0.17M) and choline chloride (40mM) was used in most experiments. Even so, the addition of KCl or other salts caused a small artifact. The magnitude of this artifact was determined in separate experiments with all the components of the reaction medium with the exception of mitochondria. In the pH traces presented below allowance has been made for this artifact, and where this has been done the curve is interrupted thus: ———| |———.

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Table 1. *Effects of gramicidin, alkali-metal ions and phosphate on succinate oxidation*

Mitochondria (9.0 mg. of protein) were suspended in a medium containing sucrose (0.20 M), tris-chloride buffer (20 mM) and succinate (tris salt) (4.17 mM) at pH 7.2. Respiratory rates were recorded with the oxygen electrode.

Additions	O ₂ uptake ($\mu\text{g. atom of O/min./mg. of protein}$)
Phosphate (1.25 mM)	0.036
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM)	0.032
Gramicidin (1 $\mu\text{g.}$) + KCl (2.5 mM)	0.038
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + LiCl (2.5 mM)	0.078
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + LiCl (5.0 mM)	0.096
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + NaCl (2.5 mM)	0.115
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + NaCl (5.0 mM)	0.144
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + KCl (2.5 mM)	0.138
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + KCl (5.0 mM)	0.154
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + RbCl (2.5 mM)	0.144
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + CsCl (2.5 mM)	0.156
Gramicidin (1 $\mu\text{g.}$) + phosphate (1.25 mM) + choline chloride (5.0 mM)	0.032

Fig. 1. Apparent K_m and V_{max} for Na^+ - and K^+ -activated succinate oxidation as a function of gramicidin concentration. Respiratory rates were determined in a medium containing succinate (4.15 mM), rotenone (0.17 μM) (to block malate oxidation), phosphate (1.25 mM), sucrose (0.17 M), choline chloride (40 mM) and mitochondria (10 mg. of protein). Buffering was provided by the phosphate and the tris used for neutralizing both phosphate and succinate. Various amounts of NaCl and KCl were added to the mitochondrial suspensions containing the amounts of gramicidin shown and the rates of respiration recorded with the oxygen electrode. The K_m and V_{max} values were determined from double-reciprocal plots. $\bullet$, K_m for Na^+ ; $\blacktriangle$, V_{max} for Na^+ ; $\circ$, K_m for K^+ ; $\triangle$, V_{max} for K^+ .

For the present investigation glutamic acid, malic acid, phosphoric acid and succinic acid were neutralized with tris base and were used as 0.5M solutions. The initial pH was 7.15 in each case and the temperature 30°. Other conditions are given in the Tables and legends to the Figures.

RESULTS

The dependence of the stimulation of succinate oxidation by gramicidin on the simultaneous presence of both phosphate and an alkali-metal ion is shown by the results in Table 1. In the presence of 0.17 μg . of gramicidin/ml. and 1.25 mM-phosphate all the alkali-metal ions tested considerably enhanced the rate of succinate oxidation. Similar results were obtained when 4 mM-glutamate plus 4 mM-malate were used as substrate. In no case was there an increase in rate of respiration when alkali-metal ion and phosphate were added in the absence of gramicidin. The dependence of the stimulation by gramicidin together with alkali-metal ion on added phosphate was not absolute and may have reflected the occurrence of endogenous phosphate in the mitochondrial preparation. Moore & Pressman (1964) have shown that Na^+ and Li^+ will not substitute for K^+ in valinomycin-stimulated succinate oxidation, and in this respect it appears that gramicidin and valinomycin differ.

No requirement for alkali-metal ion or phosphate could be demonstrated when 100 μM -2,4-dinitrophenol, 0.25 μM -carbonylcyanide-*p*-trifluoromethoxyphenylhydrazide or 17 μM -silver chloride was used to stimulate succinate oxidation.

The maximum rates of respiration at infinite alkali-metal ion concentration and the apparent Michaelis constant for alkali-metal ion, derived from double-reciprocal plots of rate of respiration against Na^+ and K^+ concentration, are plotted as a function of gramicidin concentration in Fig. 1. Approx. 30 μg . of gramicidin/g. of protein gave maximal rates of succinate oxidation at saturating concentrations of Na^+ or K^+ . However, as the gramicidin concentration was increased the apparent Michaelis constants for both Na^+ and K^+ fell.

There was a marked difference between K^+ , Rb^+ and Cs^+ on the one hand, and Na^+ and Li^+ on the other, in their effects on H^+ production and the extent to which swelling occurred in response to the addition of gramicidin. The addition of 0.17 μg . of gramicidin/ml. to a mitochondrial suspension containing potassium chloride (3.33 mM), phosphate (1.25 mM) and succinate (4.15 mM) led to an immediate swelling of the mitochondria and an appearance of H^+ in the medium. When the suspension became anaerobic (Fig. 2) or on the addition of antimycin to inhibit succinate oxidation (see Fig. 4) the mitochondria contracted and there was an alkaline pH change. The same type of

Fig. 2. Effects of gramicidin on succinate oxidation, swelling and H^+ production in the presence of K^+ . 4.15 mM-Succinate (tris salt), 1.25 mM-phosphate (tris salt), 0.17 μM -rotenone (to inhibit malate oxidation), 3.33 mM-KCl, 40 mM-choline chloride and 0.17 M-sucrose were present initially. Additions were made where indicated as follows: A, mitochondria (11.2 mg. of protein); B, 1.0 μg . of gramicidin. The upper continuous trace is a recording of respiration. The middle (broken) trace shows changes in light-scattering, a downward deflection representing swelling. The bottom continuous trace represents changes in pH, a downward deflection indicating H^+ production.

Fig. 3. Effects of gramicidin on succinate oxidation, swelling and H^+ production in the presence of Na^+ . Conditions were as given in Fig. 2 except that KCl was replaced by 3.33 mM-NaCl.

behaviour was observed when K^+ was replaced by Rb^+ or Cs^+ . When Na^+ or Li^+ was used the rate and extent of both production of H^+ and of swelling was less than that with K^+ . Again, both the pH change and swelling consequent on the addition of

gramicidin were reversed when the suspension became anaerobic (Fig. 3).

The swelling induced by K^+ , in addition to its dependence on the presence of gramicidin and phosphate, was also dependent on the occurrence, not only of electron transport through the respiratory chain, but also on coupled respiration. Thus respiratory inhibitors (antimycin with succinate as substrate, both antimycin and rotenone with glutamate plus malate as substrate) or uncoupling agents (dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine or pentachlorophenol) inhibited both the swelling and H^+ production resulting from the action of gramicidin. In contrast, oligomycin even at concentrations of $4\mu g./ml.$ was completely without effect.

Once K^+ -induced swelling had occurred it could be reversed either partially or wholly under a variety of conditions, each of which would be expected to affect energy conservation by the respiratory-chain system. As mentioned above, when the suspension became anaerobic or when antimycin was added, swelling was reversed and there was an alkaline shift of pH. These effects also occurred on the addition of the uncoupling agents dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine or pentachlorophenol. The effects of some of

these inhibitors could be reversed under appropriate conditions. Thus, when K^+ -induced swelling in the presence of succinate as substrate had been reversed by the addition of antimycin, swelling could be made to occur again by the addition of ascorbate plus tetramethyl-*p*-phenylenediamine (Fig. 4). This swelling was reversed in turn when the suspension became anaerobic. Again, when K^+ -induced swelling supported by succinate was reversed on the suspension medium becoming anaerobic, the addition of ferricyanide (1mM) caused swelling, which in turn was reversed by the addition of antimycin. These experiments indicate that the swelling observed in the presence of K^+ , gramicidin and phosphate is dependent on electron transport and can be supported by electron flux through only a restricted portion of the respiratory chain (succinate to ferricyanide, tetramethyl-*p*-phenylenediamine to oxygen).

The reversibility of the action of uncoupling agents was demonstrated by making use of the fact that bovine plasma albumin very strongly binds pentachlorophenol and not only prevents its uncoupling action (Garbus & Weinbach, 1963), but also reverses it rapidly (Chappell & Crofts, 1965b). In Fig. 5 these effects of pentachlorophenol and bovine plasma albumin are demonstrated. Albumin itself had no effect on K^+ -induced swelling.

Fig. 4. Effect of antimycin and ascorbate plus tetramethyl-*p*-phenylenediamine on respiration and swelling in the presence of gramicidin, phosphate and K^+ . Basic conditions were as given in Fig. 2. Additions were made, where indicated, as follows: A, mitochondria (10.4 mg. of protein); B, $1.0\mu g.$ of gramicidin; C, $2\mu g.$ of antimycin; D, ascorbate (2mM) together with tetramethyl-*p*-phenylenediamine (0.2mM). The upper (continuous) trace is a recording of respiration. The lower (broken) trace shows changes in light-scattering, a downward deflection representing swelling.

Fig. 5. Reversibility of the action of pentachlorophenol on the K^+ - and gramicidin-induced swelling. 4.15mM-Succinate (tris salt), 1.25mM-phosphate (tris salt), 0.17 μM -rotenone, 3.33mM-KCl, 20mM-tris-chloride buffer and 0.25M-sucrose were present initially. Additions were made where indicated as follows: A, mitochondria (9.1 mg. of protein); B, $1.0\mu g.$ of gramicidin; C, pentachlorophenol (2 μM); D, 10mg. of bovine plasma albumin. The upper (continuous) trace is a recording of respiration. The lower (broken) trace shows changes in light-scattering, a downward deflection representing swelling.

Effect of NH_4^+ . In the presence of gramicidin and phosphate, NH_4^+ , like the other univalent cations tested, caused an increased rate of respiration, the duration and linearity of which depended on the conditions used (see below). However, the effects of NH_4^+ on swelling and H^+ production were very different from those of K^+ . On the addition of

NH_4^+ to a mitochondrial suspension that had been incubated with gramicidin and phosphate a marked contraction of the mitochondria and a very small and somewhat variable alkaline shift of pH occurred. The subsequent addition of K^+ produced no swelling.

The addition of NH_4^+ to mitochondria that had been swollen in the presence of K^+ , Cs^+ or Rb^+ led to a very marked and rapid reversal of swelling, an equally rapid and large alkaline shift and a small decrease in the rate of respiration. When swelling had been produced in 3.33mM-potassium chloride, then the addition of NH_4^+ (3.33mM) produced almost complete reversal (Fig. 6). The addition of lower concentrations of NH_4^+ produced a smaller effect, but when the suspension became anaerobic the reversal of swelling was completed (Fig. 7).

The stimulation of mitochondrial respiration by NH_4^+ showed some unusual properties. The addition of ammonium chloride (3.33mM) to mitochondria suspended in the presence of succinate (4mM) (tris salt), 0.17M-sucrose and 40mM-choline chloride caused a burst of respiration that fell after a period of 20sec. to a low value. The fall in the rate of respiration coincided with the marked contraction of the mitochondria that occurred on the addition of NH_4^+ . It appeared that the decreased rate of respiration was due to an increase in the apparent Michaelis constant of the succinate dehydrogenase of the mitochondria. This aspect has not been investigated thoroughly, but support for this view is given by the fact that increasing the concentration of succinate from 4.15 to 20mM, though it did not cause a change in the rate or extent of the initial burst on the addition of NH_4^+ , caused a twofold increase in the rate after the

Fig. 6. Effect of NH_4^+ on K^+ -induced swelling and H^+ production in the presence of gramicidin and phosphate. Basic conditions were as given in Fig. 5. Additions were made where indicated as follows: A, mitochondria (9.1 mg. of protein); B, 1.0 μg . of gramicidin; C, NH_4Cl (3.33 mM).

Fig. 7. Effect of NH_4^+ concentration on K^+ -induced swelling. Conditions were as given in Fig. 6, except that the NH_4Cl concentration was varied. Additions were made where indicated as follows: A, 1 μg . of gramicidin, followed by: (a) B, NH_4Cl (1.67 mM); (b) C, NH_4Cl (0.67 mM); (c) D, NH_4Cl (0.167 mM).

burst. With K^+ in place of NH_4^+ , conditions under which the mitochondria became swollen, 2mM-succinate gave maximal rates of respiration.

Under no conditions investigated could a requirement for phosphate for NH_4^+ -stimulated respiration be demonstrated. This is in marked contrast with the behaviour with K^+ , Cs^+ , Rb^+ , Na^+ and Li^+ . This was brought out most clearly when mitochondria were suspended in a medium containing succinate (4mM), ammonium chloride (105mM) and tris-chloride buffer (20mM). The addition of 0.17 μ g. of gramicidin/ml. caused a marked increase in respiration, the rate being equivalent to that observed with K^+ and gramicidin in the sucrose-choline chloride medium. In this case the rate was maintained until the suspension became anaerobic, and no marked volume change occurred. Phosphate was completely without effect.

Effect of gramicidin together with NH_4^+ on accumulated Sr^{2+} and phosphate. Mitochondria are able to accumulate Sr^{2+} and phosphate in a respiration-dependent process similar to that used for the accumulation of Mn^{2+} and Ca^{2+} (see Chappell *et al.* 1963). In the presence of phosphate, Sr^{2+} is retained even on the addition of a respiratory

inhibitor, but the further addition of gramicidin together with NH_4^+ led to a rapid release of Sr^{2+} , as judged by the reversal of the pH shift consequent on Sr^{2+} accumulation, and the effect of the subsequent addition of ethylene glycol bis(aminoethyl)-tetra-acetate (Fig. 8) (see Chappell *et al.* 1963; Chappell & Crofts, 1965a). Neither NH_4^+ nor gramicidin was active singly. If antimycin was not added then the Sr^{2+} was not released until the suspension became anaerobic. The conditions for the release of Sr^{2+} in the presence of phosphate are, then, inhibited respiration and the presence of gramicidin together with NH_4^+ . Neither K^+ nor Na^+ would replace NH_4^+ in this respect.

The addition of dinitrophenol (100 μ M) or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (2.5 μ M) also brought about the release of Sr^{2+} in the presence of antimycin. If the respiratory inhibitor were not present then the release of Sr^{2+} did not occur until anaerobic conditions were achieved. This is illustrated in Fig. 9 for carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. In the first experiment 2 μ moles of strontium chloride were added to mitochondria suspended in the presence of 1.25mM-phosphate and 4.15mM-succinate. A rapid production of H^+ and a rapid rate of oxidation occurred. When the Sr^{2+} accumulation process had finished, H^+ production ceased and respiration fell to a low level. The addition of

Fig. 8. Effect of NH_4^+ together with gramicidin on Sr^{2+} accumulated by mitochondria. 4.15mM-Succinate, 1.25mM-phosphate, 0.17 μ M-rotenone, 40mM-choline chloride and 0.17M-sucrose were present initially. Additions were made where indicated as follows: A, mitochondria (11.7mg. of protein); B, 2 μ moles of $SrCl_2$; C, 2 μ g. of antimycin; D, NH_4Cl (3.33mM); E, 1.0 μ g. of gramicidin; F, 4 μ moles of ethylene glycol bis(aminoethyl)tetra-acetate. The upper trace is a recording of respiration. The lower trace shows changes in pH.

Fig. 9. Effect of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone on Sr^{2+} accumulated by mitochondria in the presence and absence of 0.17 μ M-rotenone. Basic conditions were as given in Fig. 2. (a) Rotenone absent; (b) rotenone present. Additions were made where indicated as follows: A, mitochondria (11.7mg. of protein); B, 2 μ moles of $SrCl_2$; C, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (2.5 μ M); D, 0.5 ml. of 5% Triton X-100. The upper trace is a recording of respiration. The lower trace shows changes in pH.

carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon (2.5 μM) caused a rapid rate of succinate oxidation initially, followed by a decline, probably as a result of accumulation of oxaloacetate (see Chappell, 1964). As the rate of respiration fell, so was the Sr^{2+} released. In the presence of 0.17 μM -rotenone the rate of oxygen consumption did not fall until anaerobic conditions were attained, and only then was Sr^{2+} released, as judged by the alkaline pH change.

In brief, the addition of (i) gramicidin together with NH_4^+ (but not K^+), (ii) dinitrophenol or (iii) carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon to mitochondria that had accumulated Sr^{2+} together with phosphate caused release of the Sr^{2+} when respiration was inhibited. The release of accumulated Mn^{2+} and phosphate under these conditions did not occur at a significant rate. When Ca^{2+} was used in place of Sr^{2+} massive mitochondrial swelling occurred on the addition of antimycin, dinitrophenol or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon alone (Chappell & Crofts, 1965a) or of gramicidin together with NH_4^+ . The Ca^{2+} was released under these conditions.

Effect of the components of the suspending medium. A variety of media have been used in the present investigation. In a medium consisting of 80mM-potassium chloride in 20mM-tris-chloride buffer, gramicidin in the presence of phosphate caused a rapid and extensive swelling and a marked stimulation of respiration. However, the swelling was not reversed spontaneously on the suspension becoming anaerobic, nor was it reversed on the addition of respiratory inhibitors or NH_4^+ . With a series of media of the compositions:

$$[\text{sucrose}] + 2[\text{KCl}] = 0.25 \text{ M}$$

and with succinate as substrate, it was shown that irreversible swelling occurred on the addition of gramicidin and phosphate when the concentration of sucrose was less than 20mM. At concentrations greater than 20mM-sucrose the swelling was progressively more completely reversed on the addition of antimycin or on the suspension becoming anaerobic.

Considerable technical difficulties were experienced in using a sensitive recording pH-meter in media containing only a low concentration of electrolyte. For this reason a medium containing choline chloride (40mM) and sucrose (0.17M) was used as a routine. At this concentration choline chloride had no effect on the swelling and respiration of liver mitochondria. With this medium the pH traces were less 'noisy' and a much smaller artifact occurred on the addition of salts. Choline chloride (80mM) together with tris-chloride buffer (20mM) alone was found to be an unsatisfactory suspending

medium, in that rates of respiration fell off with time.

The Cl^- did not appear to be necessary for the swelling, respiration and pH changes induced by gramicidin, K^+ and phosphate. In a medium containing sucrose (0.25M), succinate (4.2mM) and phosphate (1.25mM) (both as tris salts) and 0.17 μg . of gramicidin/ml., 3.3mM-potassium gluconate produced the same effects as 3.3mM-potassium chloride.

Phosphate was completely replaceable by arsenate. As shown by Moore & Pressman (1964), phosphate is not necessary for the H^+ production consequent on the addition of K^+ to mitochondria in the presence of valinomycin, and this was the case with gramicidin. Either phosphate or arsenate was necessary in order that stimulation of respiration or the swelling changes described above should occur. It was possible to separate the stimulatory effects of arsenate itself on respiration from its effects on the gramicidin system by making use of the fact that oligomycin inhibits the former process (Estabrook, 1961) but not the latter. In the presence of 0.6 μg . of oligomycin/ml. the behaviour of arsenate and phosphate was identical, i.e. the H^+ production, swelling and increase in respiration consequent on the addition of gramicidin and K^+ was indistinguishable whether phosphate or arsenate was present.

Strophanthin G (ouabain) (1 μM), which selectively inhibits the coupled active transport of Na^+ and K^+ in a variety of tissues (see Glynn, 1957), was without effect on the K^+ -gramicidin system.

DISCUSSION

Gramicidin at concentrations greater than 30 $\mu\text{g/g}$. of mitochondrial protein produced an increase in mitochondrial respiration that was dependent on the presence of K^+ and phosphate or arsenate. Gramicidin also caused the appearance of H^+ in the suspending medium; in this case neither phosphate nor arsenate was required but K^+ was. Very similar results have been reported by Moore & Pressman (1964) for the cyclic peptide valinomycin. Valinomycin and gramicidin appear to differ in the following respects: With either antibiotic, K^+ can be replaced by Rb^+ or Cs^+ , but according to Moore & Pressman (1964) Na^+ or Li^+ is not effective with valinomycin, whereas it has been found in the present investigation that Na^+ and Li^+ will replace K^+ with regard to stimulation of respiration by gramicidin, although the rate and extent of production of H^+ with Na^+ or Li^+ present were less than that with K^+ . Secondly, valinomycin acts at lower concentrations than gramicidin.

Moore & Pressman (1964) were able to show, by use of a K^+ -glass electrode, that K^+ was accumu-

lated by mitochondria in the presence of valinomycin. It seems likely that this is also the case when gramicidin is used. The swelling effects reported above are of particular interest in this respect since a volume increase occurred when gramicidin was added to mitochondria suspended in an iso-osmotic medium containing K^+ , Rb^+ or Cs^+ and to a smaller extent with Na^+ or Li^+ , together with phosphate or arsenate. It seems probable that this volume increase occurred as a consequence of the accumulation of K^+ and phosphate by the mitochondria. It is well known that swelling results in the loss of controlled respiration (Baltscheffsky, 1957; Lehninger, 1960), and it is of importance to consider therefore whether the increase in respiration caused by addition of gramicidin, K^+ and phosphate is consequent on the swelling, or even whether the swelling is caused by the uncoupling action of gramicidin. The first alternative seems unlikely since, when NH_4^+ was used in place of K^+ , contraction of the mitochondria was observed and yet the respiration was increased to the same extent as when K^+ was used. The second alternative, i.e. that swelling is a result of a chemical uncoupling action of gramicidin (together with K^+) is rendered unlikely, since under similar conditions other uncoupling agents, e.g. dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine or pentachlorophenol, did not cause significant volume changes.

It seems most likely therefore that the gramicidin-stimulated increase in respiration, the H^+ production and volume changes are consequent on the accumulation of K^+ by mitochondria, as suggested by Moore & Pressman (1964) for valinomycin. However, if it is assumed that gramicidin and

valinomycin act in essentially the same way, it seems unlikely that the suggestion of those authors, that valinomycin activates a K^+ pump in the mitochondrial membrane, is correct. The ability of gramicidin and NH_4^+ to cause, in the presence of antimycin, the release of Sr^{2+} accumulated by mitochondria could hardly be due to activation of a pump, but is much more likely to be due to an increased permeability of the mitochondrial membrane to NH_4^+ .

Most of the observations made by Moore & Pressman (1964), and in the present paper, may be accounted for if the following assumptions are made: (a) that the mitochondrial membrane is limitedly permeable to both univalent anions (e.g. Cl^-) and cations, and in this respect resembles many cell membranes other than the erythrocyte membrane; (b) that there is a respiration-dependent H^+ pump, which results in the mitochondria being more alkaline than the medium in which they are suspended (cf. Mitchell, 1962); (c) that gramicidin and valinomycin cause an increase in the rate at which K^+ and other univalent cations are able to pass across the mitochondrial membrane.

The extent to which each of these assumptions is valid is not known. However, mitochondria do not swell in iso-osmotic potassium chloride solutions, and it follows therefore that they are impermeable to K^+ or to Cl^- , or to both. Impermeability to K^+ and especially NH_4^+ is indicated by the fact that gramicidin apparently increases the penetration of the mitochondrial membrane by these ions. Impermeability to Cl^- is indicated by the work of Robertson, Wilkins, Hope & Nestel (1955) on plant mitochondria, and to both K^+ and Cl^- by the work of Amoore (1960) on liver mitochondria. Some

Scheme 1. Possible mechanism by which electron transport and ion movements are related in mitochondria. It is visualized that electron transport causes the production of a non-phosphorylated 'high-energy' intermediate ($X \sim I$), which, when it is decomposed, leads to the ejection of H^+ by the pump 1. This pump may in turn be coupled with the transport of Sr^{2+} (or Mn^{2+} or Ca^{2+}) by pump 2, or with the entry of K^+ through the mitochondrial membrane when gramicidin is present, possibly through a pore labelled 3. Pump 4 allows OH^- to be exchanged for either phosphate or arsenate.

evidence for a respiration-dependent H^+ pump in mitochondrial as well as bacterial membranes has been presented by Mitchell (1962). A great body of evidence has been presented that the cyclic-peptide antibiotics tryocidin, gramicidin and polymyxin are surface-active agents that cause increased permeability of the bacterial protoplast membrane (see Newton, 1962), and it is not unreasonable to suppose that this would apply to the mitochondrial membrane.

The following sequence of events may be visualized in relation to the hypothesis outlined above (see Scheme 1). Mitochondria suspended in, e.g. the sucrose-choline chloride medium, by virtue of the presence of the postulated respiration-dependent H^+ pump, will tend to eject H^+ , but will be unable to do this to any extent because there is no equivalent positively charged ion present to take its place. However, on the addition of K^+ and gramicidin, K^+ is able to enter in exchange for H^+ , which accounts for the acid production in the medium, but this will not lead to marked stimulation of respiration, because this exchange is not a process that requires energy, and would in any case be limited by the net negative charge within the mitochondria. Swelling does not occur because there has been no change in osmotic pressure. However, on the addition of phosphate (or arsenate) $H_2PO_4^-$ enters the mitochondrion (possibly in exchange for OH^- since it has been postulated that the interior of the mitochondrion is alkaline) and thus provides more H^+ to be pumped out in exchange for K^+ . Now there has been an increase in internal osmotic pressure (due to accumulation of K^+ and phosphate) and the mitochondria swell. An increase in respiration occurs because energy is required for the net uptake of electrolyte. The immediate cause of the increased rate of respiration would be the energy requirement for the H^+ pump.

The results obtained with NH_4^+ may also be interpreted in terms of the postulates outlined above. The addition of gramicidin to mitochondria suspended in buffered iso-osmotic ammonium chloride solution did not cause swelling. It is postulated that this is so because, though the mitochondria are now permeable to NH_4^+ as a result of the action of gramicidin, they are still impermeable to Cl^- . However, there is marked phosphate-independent stimulation of respiration because NH_4^+ is exchanged for H^+ , activating the H^+ pump. This is followed by rapid dissociation of NH_4^+ to ammonia and H^+ , aided by the alkaline environment of the mitochondria, and the ammonia, since it is uncharged, may now diffuse from the mitochondria. The H^+ may be used to further activate the H^+ pump and bring in more NH_4^+ in exchange. Phosphate is not required in this case because NH_4^+ provides H^+ in the mitochondrion by dis-

sociation, whereas, with K^+ , $H_2PO_4^-$ (or arsenate) fulfils this function. No swelling with NH_4^+ occurred under these conditions, presumably because there was no net increase in internal osmotic pressure.

The effect of NH_4^+ in reversing K^+ -induced swelling in the presence of gramicidin in the sucrose-choline chloride medium is also interpreted in terms of the hypothesis outlined above. When NH_4^+ exchanges for K^+ inside the mitochondria, the NH_4^+ will dissociate, effectively acidifying the inside of the mitochondrion, leading to loss of $H_2PO_4^-$, a net decrease of internal osmotic pressure and consequent contraction. The contraction observed under anaerobic conditions and on the addition of respiratory inhibitors would also be due to a loss of K^+ and phosphate, since the H^+ pump would no longer be active.

The discharge of accumulated Sr^{2+} and phosphate by NH_4^+ and gramicidin could also be accounted for in similar terms. Indeed the uptake of the bivalent cations Mn^{2+} , Sr^{2+} and Ca^{2+} may be accounted for in terms of this hypothesis. The bivalent cations would enter the mitochondria on a specific carrier in exchange for H^+ generated by

Fig. 10. Relationship between Sr^{2+} accumulation and the gramicidin effect. 4.15 mM-Succinate (tris salt), 0.17 μ M-rottenone, 40 mM-choline chloride and 0.17 M-sucrose were initially present. Additions were made where indicated as follows: A, mitochondria (10.5 mg. of protein); B, 2 μ moles of $SrCl_2$; C, KCl (3.33 mM); D, 0.5 μ g. of gramicidin; E, phosphate (1.25 mM); F, NH_4Cl (3.33 mM). The upper continuous trace is a recording of respiration. The middle (broken) trace shows changes in light-scattering, a downward deflection representing swelling. The bottom continuous trace shows changes in pH.

the H^+ pump. In the presence of phosphate, $H_2PO_4^-$ would enter (in exchange for OH^-) and interaction of bivalent cation and phosphate would result in the precipitation of metal phosphate (e.g. manganese phosphate) that is observed. This would lead to production of H^+ within the mitochondria that would activate the H^+ pump. The releasing effect of NH_4^+ and gramicidin again would be due to the effective acidification of the mitochondrial interior by dissociation of NH_4^+ to ammonia and H^+ . The presence of a respiratory inhibitor or anaerobic conditions is required because the activity of the bivalent-metal-ion pump exceeds that of the gramicidin-dependent univalent-cation system. Support for this view is given by the experiment shown in Fig. 10. Strontium chloride ($2\mu\text{moles}$) was added to mitochondria suspended in a phosphate-free sucrose-choline chloride medium containing succinate as substrate. The pH trace indicates that some of the Sr^{2+} was accumulated by the mitochondria, and that a steady state was achieved in which respiration was at a level higher than that of resting mitochondria, possibly as a result of the active influx and passive efflux of Sr^{2+} . The addition of potassium chloride and gramicidin was without effect on any of the parameters measured. The failure of gramicidin to elicit H^+ production, which would have occurred in the absence of Sr^{2+} , was presumably due to the fact that Sr^{2+} had already done this. However, on the addition of phosphate, Sr^{2+} was rapidly accumulated (large pH shift) and respiration was stimulated, but the mitochondria did not swell (indicating that K^+ had not entered the mitochondria) until the Sr^{2+} -induced pH shift had ceased. Only then did the K^+ -induced swelling occur. On the suspension becoming anaerobic contraction occurred (indicating loss of K^+), and there was a small pH shift, presumably for this reason. On adding NH_4^+ there was a large pH shift due to release of Sr^{2+} , presumably by the mechanism postulated above.

Further support for the view that NH_4^+ in the presence of gramicidin acts as an acidifying agent of the interior of the mitochondria is that dinitrophenol and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone acted in the same way as NH_4^+ together with gramicidin on Sr^{2+} and phosphate release. As Mitchell (1962) has emphasized, these agents are potential lipid-soluble H^+ carriers and could therefore act in the same way as is postulated for NH_4^+ in the presence of gramicidin.

There is a striking similarity in action between 'classical' uncoupling agents (dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) and gramicidin in the presence of NH_4^+ , or of phosphate together with uni- or bi-valent cations. It is tentatively suggested that the concept of an outwardly directed H^+ pump, which causes the interior of the mitochondrion to be more alkaline than the suspending medium, is a reasonable way to account for the phenonoma described in the present paper. The further postulate that the mitochondrial membrane is normally relatively impermeable to all ions and that specific 'permeases' exist for each substance transferred across the mitochondrial membrane, e.g. the bivalent-cation-transferring system or the gramicidin-activated system for univalent ions, enables one to account for a large number of facts.

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