Passive Ion Permeability of the Chromaffin-Granule Membrane

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'Ghosts' of bovine chromaffin granules, in which the complex mixture of proteins and solutes normally found in the granule matrix is replaced by buffered sucrose, are osmotically sensitive. They shrink when the osmotic pressure of the suspension medium is increased, and swell if solute entry is facilitated by the addition of ionophores. Swelling in the presence of ionophores has been used to investigate the passive ion permeability of these membranes. They have a very low permeability to K^+ ions (of the order of 10^{-10} cm/s); their permeability to protons, Na^+ and choline ions is too low to be detected by these methods. Their passive permeability to anions decreases in the order:

the permeability to thiocyanate being of the order of 10^{-7} cm/s. Coupled proton and anion entry is extremely slow, except for weak acids. Fluoride, unexpectedly, also appears to enter rapidly when proton/ K^+ exchange is facilitated by nigericin. In the presence of K^+ salts, nigericin, like valinomycin, induces lysis of intact granules, an effect that is not dependent on the presence of a permeant anion, but is dependent on the pH gradient across the membrane.

The chromaffin granule of the adrenal medulla is a membrane-bound secretory granule containing catecholamine, ATP and protein in high concentrations (Hillarp, 1959). It is known that catecholamines are accumulated within these granules by an active process which utilizes ATP (Taugner, 1971; Phillips, 1974b). The granules also contain other ions, such as Ca^{2+} and Mg^{2+} (Borowitz *et al.*, 1965) and K^+ (Johnson & Scarpa, 1976a), but the mechanisms by which these, and ATP, are accumulated (or passively sequestered) within the granules are unknown. In the present paper I report a study of the passive ion permeability of the bovine chromaffin-granule membrane, information that is required before alternative mechanisms of solute transport across the membrane can be assessed.

The membranes can be isolated in the form of resealed 'ghosts' (Phillips, 1974a); I show that these are osmotically sensitive, and use this preparation to investigate ion permeability by the light-scattering methods that have been successfully applied to mitochondria (Chappell, 1968).

A study has been made of the permeability of chromaffin-granule membranes by using light-scattering, potentiometric and spectrophotometric studies of intact granules (Johnson & Scarpa, 1976b); my observations on the isolated membranes confirm and extend these studies. Several other studies have also provided information about the permeability of the membrane: thus Dolais-Kitabgi & Perlman (1975) showed that valinomycin-induced lysis of granules in media containing K^+ salts is dependent on

the anion present, and this is also the case for MgATP-induced lysis of granules in iso-osmotic salts media (Casey *et al.*, 1976). Studies on intact granules can be performed very easily by following the absorbance change that accompanies lysis (Trifaró & Poisner, 1967) and concomitant release of matrix components to the medium (Morris *et al.*, 1977). This method has the disadvantage, however, that factors other than osmotic changes may initiate the lytic event, for example entry of a solute that disrupts the high-molecular-weight catecholamine-storage complex; such problems can be overcome by the use of 'ghosts', as described in the present paper.

Experimental

Materials

2H_2O was from Norsk Hydro, Oslo, Norway; Hepes* and Mes were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Ionophores, (generously given by Dr. D. K. Apps of this Department) were from Calbiochem, Bishops Cleeve, Herts., U.K. (valinomycin), and Eli Lilly, Indianapolis, IN, U.S.A. (nigericin). Other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

'Buffered sucrose' contained 10 mM-Hepes, adjusted to pH 7.0 with NaOH.

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; ATPase, adenosine triphosphatase.

Methods

Preparation of bovine chromaffin-granule 'ghosts'.

The basic method of preparation and the materials used have been described previously (Phillips, 1974a). The only difference was that 'ghosts' were prepared on a larger scale than previously, by increasing the size of the Sephadex column used for lysis to 10 cm $\times$ 2.4 cm (diam.). The crude 'ghosts' (7 ml portions) were purified by gradient centrifugation through buffered 0.4 M-sucrose (4.5 ml) above 3.0 ml of 0.3 M-sucrose in $^2\text{H}_2\text{O}$ in glass Corex tubes (Beckman, Palo Alto, CA, U.S.A.). Centrifugation was in an angle rotor at 40000g for 30 min at 4°C.

Swelling experiments. Experiments were performed at room temperature (22°C). 'Ghosts' at this temperature were added to 0.3 M-sucrose buffered with 10 mM-Hepes (in some experiments 50 mM-Hepes was used) (final volume, 2 ml; final concentration, 0.1–0.2 mg/ml). Their A_{540} (approx. 0.10–0.20) was examined in a Gilford-modified Unicam SP.500 spectrophotometer (Gilford model 252) with a recorder. Additions were made directly to the cuvette. Salt solutions added were 2.0 M (0.5 M for K_2SO_4 and Na_2SO_4); ionophores (in ethanolic solution) were added in a volume of 10 μl . The tracings of chart recordings shown in the Figures have been corrected for volume changes on the addition of salt solutions.

Swelling experiments with intact granules were performed by adding a suspension of well-washed 'crude' granules (Phillips, 1974a) to 0.3 M-sucrose buffered with 50 mM-Hepes, to give an A_{540} of approx. 0.8.

Results

Osmotic sensitivity of 'ghosts'

It has been shown that the reciprocal of the absorbance of mitochondrial suspensions is a linear function of mitochondrial volume as long as the refractive index of the medium is constant (Tedeschi & Harris, 1958). The absorbance of suspensions of 'ghosts' was therefore examined as a function of osmotic pressure in an experiment in which KCl (the refractive index of which differs rather little from that of water over the concentration range used) was added to a 'ghost' suspension in buffered sucrose. Fig. 1(a) shows that 'ghosts' shrink, as shown by an increase in A_{540} , as the osmolarity is increased, and they appear to obey the Boyle-van't Hoff law for osmometers, as shown by plotting $1/A$ as a function of reciprocal osmolality (Fig. 1b). The osmotic 'dead space' is given, in terms of A_{540} of the suspension, by the intercept on the ordinate.

The shrinking of the 'ghosts' is reversible by the addition of ionophores, which affect the absorbance in a predictable way. In Fig. 2(a) is shown swelling of 'ghosts' induced by trimethyltin and nigericin. The order of addition of these ionophores is unimportant, but both are required; in this case, trimethyltin catalyses Cl^-/OH^- antiport (Selwyn *et al.*, 1970), and nigericin catalyses K^+/H^+ antiport. The 'ghosts' therefore swell rapidly in 50 mM-KCl until the ionic concentration gradients are eliminated. Nigericin alone induces swelling in 50 mM-potassium acetate (Fig. 2b), since acetate enters as the uncharged, protonated form. Valinomycin (catalysing K^+ uniport) plus uncoupler (carbonyl cyanide *p*-

Fig. 1. Osmotic sensitivity of resealed chromaffin-granule 'ghosts'

(a) 'Ghosts' (approx. 1 mg of protein/ml) were suspended in buffered 0.35 M-sucrose; additions of KCl were made to the concentrations shown. The resulting A_{540} has been corrected for the dilutions of the sample. (b) Reciprocal of the A_{540} values shown in (a) were plotted as a function of the reciprocal of the osmolality of the suspension medium. Molarities were converted into osmolalities by using the data in *C.R.C. Handbook of Chemistry and Physics* (1974), 55th edn.

Fig. 2. Swelling of chromaffin-granule 'ghosts' induced by ionophores

'Ghosts' were suspended in buffered sucrose and their A_{540} recorded. (a) Addition of KCl to 50mM was followed by trimethyltin chloride (TMT; 0.5mM) and nigericin (NIG; 35nM). (b) Addition of potassium acetate (KOAc) to 50mM was followed by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 5 μ M), valinomycin (VAL; 0.5 μ M) or nigericin (NIG; 35nM). (c) Addition of sodium acetate (NaOAc) or potassium phosphate (KP) to 50mM was followed by nigericin (NIG; 35nM).

trifluoromethoxyphenylhydrazone) can replace nigericin, as expected. The traces fail to return to the baseline during swelling; this may be due to the altered refractive index of the medium. However, some preparations of 'ghosts' seemed to be more sensitive to ionophores than others, although results were always qualitatively similar (compare Figs. 2a and 5, for example).

Purity of the 'ghost' suspension

The purity of chromaffin-granule 'ghosts' prepared as in this work has been assessed elsewhere (Phillips, 1974a), and it is certain that they are the major contributor to the optical effects described. It is unlikely that mitochondria, for instance, are significant contaminants. In the absence of nigericin, 'ghosts' do not swell significantly in sodium acetate

Fig. 3. Swelling of chromaffin-granule 'ghosts' in K^+ salts media induced by valinomycin

'Ghosts' in buffered sucrose were shrunk by addition of the K^+ salt shown, to a concentration of 50 mM; 0.5 μ M-valinomycin was added at the times indicated by the arrows. Absorbance changes were followed at 540 nm.

or potassium acetate solutions (Figs. 2b and 2c), showing the absence of a Na^+/H^+ or K^+/H^+ antiport [Mitchell & Moyle (1969) have shown the former to be characteristic of rat liver mitochondria under these conditions]. Addition of nigericin to 'ghosts' in sodium acetate catalyses a very slow Na^+/H^+ antiport (Fig. 2c). In addition, 'ghosts' do not swell in potassium phosphate (pH 7) nigericin has very little effect (Fig. 2c) and we may conclude that mitochondrial phosphoric acid translocation (Mitchell & Moyle, 1969) is absent.

Anion permeability of chromaffin-granule membranes

Passive anion permeability of the membranes was investigated by permitting the ghosts to shrink in a buffered sucrose solution containing a K^+ salt (50 mM in all cases). The absorbance increase induced by adding the salt solution varied considerably, presumably because of their different refractive indices. Valinomycin was added (Fig. 3), permitting K^+ entry in response to a potential difference (inside negative) induced by anion entry. The rate of anion entry was found to decrease in the order:

The anions at the end of this series appeared to be essentially impermeant.

Passive permeability to cations and protons

Passive cation permeability can be tested by shrinking the 'ghosts' with a solution of a chloride,

Fig. 4. Effect of cations on chromaffin-granule 'ghost' swelling

'Ghosts' in buffered sucrose were shrunk by addition of the salt solution shown, to a concentration of 50 mM. At the times shown by the arrows, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 5 μ M) and trimethyltin chloride (TMT; 0.5 mM) were added. Absorbance changes were followed at 540 nm.

and adding trimethyltin with an uncoupler. Under these conditions the Cl^- concentration gradient is used to set up a potential gradient (inside negative), with swelling dependent on electrophoretic cation entry. The membrane was found to be impermeable to Na^+ and choline ions, but slightly permeable to K^+ ions (Fig. 4). The latter is not due to K^+/H^+ antiport: the slight swelling observed is not found with trimethyltin alone (Fig. 2a).

The proton permeability of the membrane is low. The 'ghosts' fail to swell in response to a K^+ potential gradient induced by valinomycin in potassium acetate (Fig. 5); charged acetate ions do not enter, and entry of uncharged acetic acid molecules is impossible because protons fail to leak across the membrane. Similarly, addition of trimethyltin to 'ghosts' in a medium containing KCl and valinomycin fails to accelerate swelling (Fig. 5). In KCl with valinomycin the swelling rate is dependent on the rate of passive Cl^- entry; an acceleration of swelling rate on adding trimethyltin would imply that OH^- , exchanged for Cl^- , was able to leak back rapidly across the membrane. This is not found, and we conclude that passive permeability to OH^- is less than that to Cl^- . However, subsequent addition of uncoupler leads to rapid swelling, as would be expected.

Passive anion/ OH^- exchange

Fig. 5 also shows that passive Cl^-/OH^- antiport is absent, or at least slower than K^+ entry; this is shown by the fact that addition of uncoupler fails to stimulate swelling induced in KCl solution by the addition of valinomycin. It should be noted that, in this

Fig. 5. Proton permeability of chromaffin-granule 'ghosts'. 'Ghosts' in buffered sucrose were shrunk by addition of 75mM-KCl or 75mM-potassium acetate and the absorbance changes followed at 540nm. At the times shown by the arrows, valinomycin (VAL; 0.5 μ M), carbonyl *p*-cyanide trifluoromethoxyphenylhydrazone (FCCP; 5 μ M) or trimethyltin chloride (TMT; 0.5mM) was added.

context, one cannot distinguish experimentally between anion/ OH^- exchange and entry of protons in association with an anion (H^+ /anion symport).

A systematic test was made, similar to that shown for anion entry in Fig. 3, for anion/ OH^- exchange: 'ghosts' were shrunk in solutions of K^+ salts, and nigericin (catalysing K^+/H^+ antiport) was added at zero time. In a similar experiment, Mitchell & Moyle (1969) showed that rat liver mitochondria would swell slowly when nigericin was added to suspensions in KCNS and K_2SO_4 , but not in KCl. As expected, chromaffin-granule 'ghosts' swell rapidly (Fig. 6) on adding nigericin to a suspension in potassium acetate. Nigericin permits exchange of K^+ ions for the protons accumulated inside the 'ghosts' as a consequence of the entry of the uncharged acetic acid molecule. There is also fairly rapid swelling in KHCO_3 , in which case electroneutral CO_2 entry effectively transports a proton to the interior (Lehninger, 1974). Although there was slight swelling in the presence of KCNS (perhaps due to HCNS entry), swelling in the presence of a number of other anions, including sulphate, was negligible.

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Fig. 6. Swelling of chromaffin-granule 'ghosts' induced by nigericin

'Ghosts' in buffered sucrose were shrunk by addition of the salt solution shown, to a concentration of 50mM, followed by nigericin (NIG; 35nM), or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 5 μ M) and valinomycin (VAL; 0.5 μ M). Absorbance changes were followed at 540nm.

Quite unexpected, however, was the finding that 'ghosts' suspended in 50mM-KF swell fairly rapidly on adding nigericin. This suggests that there is a linked H^+/F^- entry or F^-/OH^- exchange across the membrane. Consistent with this is the effect of uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) on 'ghosts' in KF (Fig. 6): there is slow swelling, presumably limited by the rate of passive K^+ entry in response to a gradient of F^- ions. Swelling is just detectable on adding nigericin to 'ghosts' in NaF. The 'ghosts' do not swell in the absence of nigericin, since there is no passive K^+ or Na^+ exchange for protons across the membrane.

Experiments with intact chromaffin granules

Several studies have been made of the effects of ionophores on intact chromaffin granules. Such studies complement investigations of the properties of the membranes by providing information about ion concentrations within the intact granule. Inferences are necessarily indirect, however, since measurements are made of granule lysis: although osmotic swelling may well lead to lysis, other effects on the complex core of the granules may lead to the same result.

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Fig. 7. Effects of nigericin and valinomycin on intact chromaffin granules

Chromaffin granules were suspended in the salt solutions shown [150 mM, apart from K₂SO₄, which was 75 mM in the presence of 25 mM-Na₂SO₄ in (a) and 100 mM in (b)], buffered with 50 mM-Hepes, pH 7.0. Absorbance was followed at 540 nm. All traces have been adjusted to an initial absorbance of 100% and have been corrected for the slight decrease produced by addition of ethanol (used as solvent for nigericin and valinomycin, at 5 μl/ml). (a) Absorbance change resulting from addition of 35 nM-nigericin. (b) Absorbance change resulting from addition of 0.5 μM-valinomycin. Note that the absorbance scale differs in (a) and (b).

Fig. 8. Effect of pH on nigericin-induced chromaffin-granule lysis

Chromaffin granules were suspended in 0.1 M-K₂SO₄ buffered with 20 mM-Mes at the pH shown. Absorbance changes were recorded at 540 nm, after the addition of 35 nM-nigericin. The initial rate of absorbance decrease is shown as a function of the pH of the suspending medium.

Granule lysis is conveniently followed experimentally by recording a decrease of light-scattering (Trifaró & Poisner, 1967); this is because the major contributor to the scattering by a granule suspension is the core material, which has an extremely high refractive index. In the course of a study of the effects of various agents on intact granules, the ionophore nigericin was found to have a dramatic effect on the absorbance of granule suspensions (Fig. 7). This effect, which, from its extent, is likely to reflect granule lysis, was indeed found to be dependent on the osmotic pressure of the suspending medium. This was shown by adding sucrose to suspensions of the granules in 0.1 M-K₂SO₄: 0.1 M-sucrose almost completely eliminated the nigericin effect, intermediate rates being found at lower sucrose concentrations.

Nigericin had no effect if K⁺ was totally replaced by choline. Granules suspended in choline chloride media containing only 10 mM-KCl lysed quite rapidly, however, on addition of nigericin.

Chromaffin-granule 'ghosts' (i.e. sealed membranes lacking core material) fail to swell on the addition of nigericin to suspensions in media containing most K⁺ salts (as discussed above) and so it seemed unlikely that lysis was due solely to solute entry, as it is after addition of valinomycin (Dolais-Kitabgi & Perlman, 1975). Rather, an effect on the structure of the granule core seemed to be implicated. The results of an experiment on variation of the pH of

the supporting medium for nigericin-induced lysis are shown in Fig. 8. The effect is highly pH-dependent, presumably reflecting the magnitude of the pH gradient across the membrane. Nigericin promotes K^+/H^+ exchange, and it is known that the interior of the intact granules is maintained at about pH 5.5 (Johnson & Scarpa, 1976a; Casey *et al.*, 1977).

The lytic effect of nigericin was relatively independent of the anion present. In Fig. 7(a) the effects of nigericin are shown in the presence of a K^+ concentration of 150 mequiv./litre with four anions; this may be contrasted with valinomycin-induced lysis in Fig. 7(b), in which the passive permeability of the anion determines the rate of lysis. In this case, by contrast with the effect of nigericin, valinomycin induces lysis at rates that vary over at least two orders of magnitude.

Discussion

Passive swelling of 'ghosts'

The chromaffin-granule 'ghosts' shrink in a predictable way on the addition of salt solutions, and can be caused to swell (as measured by light-scattering) by addition of ionophores. They obey the Boyle-van't Hoff Law for osmometers over a range of solute concentrations (Fig. 1). At high KCl concentrations, passive solute entry and swelling of the 'ghosts' complicates the traces: the experiment was performed rapidly to minimize the effect of this, but deviation from ideal behaviour was found when the concentration of KCl added to the 'ghosts' (in 0.35 M-sucrose) exceeded 0.15 M.

The membrane is impermeable (within the limits of these experiments) to protons, Na^+ and choline ions; it is slightly permeable to K^+ ions. Anion permeability follows the lyotropic series (Tasaki *et al.*, 1965). In previous studies (Dolais-Kitabgi & Perlman, 1975; Casey *et al.*, 1976), ion permeabilities have been inferred from the results of granule-lysis experiments, and the good agreement between different methods is reassuring. Thus valinomycin induces lysis of granules suspended in media containing the K^+ salts of permeant anions (Dolais-Kitabgi & Perlman, 1975), since rapid solute entry is followed by water entry. Taugner (1972) showed that, in the absence of ionophore, the nature of the anion was the main factor determining efflux of catecholamines from intact granules (leakage across the membrane and granule lysis were not distinguished): for granules suspended in buffered 0.16 M solutions of salts, efflux rates increased in the order:

For K^+ salts, slow K^+ entry down its concentration gradient would be accompanied by anion entry at a

rate dependent on the entry of the slower ion. However, other effects may be important when considering intact granules: for example, catecholamine leakage and consequent disruption of the core structure may be promoted by certain ions.

An unexpected finding to emerge from the present study is the nigericin-induced swelling of 'ghosts' suspended in a medium containing 50 mM-KF (Fig. 6). This occurs at about one-quarter of the rate of nigericin-induced swelling in 50 mM-potassium acetate (Figs. 2b and 6) and is similar to the rate in $KHCO_3$. It suggests that some mechanism exists whereby F^- and OH^- ions may exchange across the membrane: the swelling is considerably faster than that in KCNS or K_2SO_4 (both HCNS and H_2SO_4 have been found to be translocated non-electrogenically across mitochondrial membranes by Mitchell & Moyle, 1969). HF translocation cannot, of course, be distinguished in these experiments from F^-/OH^- exchange.

Calculation of ion fluxes

It is possible to make a very approximate calculation of the ion fluxes across the 'ghost' membranes, and hence of their permeabilities, from the data presented. To do this, one must assume that the absorbance increase on adding a salt solution is entirely due to osmotic shrinkage, and that a re-swelling to give the original absorbance would correspond to an entry of salt such that its concentration inside the 'ghost' is the same as that outside. A rough measure of the rate of entry of an ion can be obtained from the initial rate of decrease of absorbance, from the plots in the present paper (e.g. Fig. 4 for K^+ ions and Fig. 3 for anions). It can easily be calculated that a spherical chromaffin granule (diameter 300 nm) has a surface area of $20 \mu m^2/\mu m^3$. The 'water space' of granule preparations, as measured by 3H_2O exchange (Phillips *et al.*, 1977), is $4.3 \mu l/mg$ of protein. Taking a value for the total internal space of $5 \mu l/mg$, and assuming that 80% of the granule protein is released on lysis (Winkler, 1976), a membrane area of about $5000 cm^2/mg$ of protein is obtained for the 'ghosts'.

The 'ghost' preparations have an internal volume of about $4 \mu l/mg$ of protein (J. H. Phillips, unpublished work), and I assume that most of the membrane present is in the form of osmotically active vesicles. The initial rates of entry of ions can then be calculated as fluxes across membranes of known area. Although such calculations are obviously very approximate, a K^+ flux can be derived from Fig. 4 of about 2×10^{-14} g-ion/ cm^2 per s at an external K^+ concentration of 0.05 g-ion/litre. This corresponds to a permeability (flux/concentration) of about 4×10^{-10} cm/s, ignoring potential-difference effects. This value is remarkably close to the value for the passive K^+ ion permeability of low- K^+ sheep ery-

throcyte membranes (5.7×10^{-10} cm/s) calculated by Stein (1967, p. 99) and the value for human erythrocyte membranes (2.4×10^{-10} cm/s) quoted by Jain (1972, p. 143).

Na^+ and choline permeabilities are too low to be measured. The Cl^- ion permeability is about 2–5 times the K^+ ion value, a permeability of 7×10^{-10} cm/s being obtained from Fig. 3; this may be compared with the value of 3×10^{-8} cm/s for the passive Cl^- ion permeability of erythrocyte membranes that can be calculated from the data of Hunter (1971). The CNS^- ion is about 50–100 times as permeant as the Cl^- ion, a value of about 7×10^{-8} cm/s being obtained from Fig. 3.

These values for passive ion permeability are remarkably low. They are interesting in view of the fact that the chromaffin-granule membrane is unusual among intracellular membranes in having a particularly high cholesterol content, a ratio of 0.69 mol of cholesterol/mol of phospholipid being given by Winkler *et al.* (1970). Johnson & Scarpa (1976b) have determined the proton permeability of the membranes by titration, obtaining a value of $0.088 \mu\text{g-ions/s per pH unit per g of protein}$, equivalent to 10^{-13} g-ions/cm² per s per pH unit; Mitchell & Moyle (1967) obtained a value of 2.75×10^{-13} g-ions/cm² per s per pH unit for the rat liver mitochondrial membrane.

Lysis of intact granules induced by nigericin and valinomycin

Dolais-Kitabgi & Perlman (1975) showed that valinomycin induces lysis of intact chromaffin granules suspended in iso-osmotic solutions of K^+ salts, as long as these contain a permeant anion; they inferred a permeability sequence:

The mechanism of lysis is presumably water entry consequent on the increased internal osmotic pressure after solute entry. Such effects can easily be observed by light-scattering by the method of Trifaró & Poisner (1967; see Fig. 7b).

An unexpected finding was the observation that nigericin has a similar lytic effect in K^+ salts media (Fig. 7a), since an electroneutral exchange of K^+ for H^+ does not lead to swelling of 'ghosts'. The explanation is presumably that there is a rapid efflux of protons from the interior of the granules when nigericin is added in the presence of a high concentration of K^+ ions. The interior of the granule is powerfully buffered at about pH 5.5 (Johnson & Scarpa, 1976b; Casey *et al.*, 1977), so that electro-neutral K^+ entry will lead to an increase in osmotic pressure and consequently lysis, since the K^+ ions are presumably not bound. It is also possible that the pH

gradient itself is obligatory for granule integrity. A reservoir of protons inside the granules is formed by the high concentration (up to 210 mg/ml; Phillips *et al.*, 1977) of acidic proteins with isoelectric points in the region of pH 5 (Baumgartner *et al.*, 1974).

In accord with this interpretation is the effect of pH, shown in Fig. 8. As the pH of the medium is lowered, the pH gradient across the membrane decreases, and the rate of lysis decreases. Nevertheless, some lysis occurs at pH 5.5; the high concentration of K^+ ions in the medium (200 mM) may be able to drive an outward movement of protons in the absence of a pH gradient, or even in the presence of a small gradient of opposite sign (outside acid); alternatively, the internal pH may decrease as the external pH is lowered, as is the case with lysosomes (Reijngould *et al.*, 1976) if membrane permeabilities increase at lower pH values.

The rate of lysis with nigericin varies to some extent with the anion (Fig. 7a), but this bears no relation to its permeability, and cannot at present be explained. These effects are of quite a different order of magnitude to those found with valinomycin, however (Fig. 7b).

Composition of the intact chromaffin granule

The concentrations of certain components within chromaffin granules are known; others are uncertain. We have determined the internal volume of chromaffin granules (Phillips *et al.*, 1977) and have calculated that in their interior, protein, catecholamine, ATP, calcium and magnesium are present in the following concentrations: 210 mg/ml, 0.71 M, 0.16 M, 0.03 M and 0.006 M respectively. Many other components are likely to be present in millimolar concentrations (Winkler, 1976). Complexes of molecules within the matrix must be in equilibrium with free components in solution within the granule interior, equilibration across the membrane being maintained partly by diffusion (Donnan equilibrium) and partly by ion pumps.

We do not know the free concentrations (activities) of these components inside the granules, nor do we know whether the granules contain the common ions found within the cytoplasm. Furthermore it is clear that ions such as K^+ and Cl^- are likely to be lost from the granules during the course of the conventional purification in sucrose media at low ionic strength unless the permeability of the membrane to these ions is much decreased at low temperatures, as it is to sugars (Perlman, 1976). Lysosomal membranes undergo changes of permeability with temperature, becoming more permeable to Na^+ and K^+ ions at low temperatures (Reijngould & Tager, 1975).

A low internal concentration of free K^+ ions (perhaps 5 mM or less) and extremely low concentrations of free Ca^{2+} and Mg^{2+} ions can be inferred from the studies of Johnson & Scarpa (1976b) with

ionophores. The free concentrations of the latter ions are probably below $25\ \mu\text{M}$ (although it is uncertain to what extent proton efflux can drive ion uptake in studies with ionophores), indicating that the interior of the granule contains high-affinity binding sites for these ions.

There is clearly a marked similarity between chromaffin granules and lysosomes: both contain a high concentration of proteins and maintain a low internal pH. However, lysosomal membranes are thought to be relatively permeable to protons at 25°C , because of a H^+ /anion symport or OH^- /anion antiport (Reijngould & Tager, 1975), and therefore to maintain their low internal pH by establishment of a Donnan equilibrium. In the chromaffin granule, the low internal pH appears to be maintained by a proton-translocating ATPase (Bashford *et al.*, 1976; Casey *et al.*, 1977).

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