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FACTORS AFFECTING THE SUSCEPTIBILITY OF STAPHYLOCOCCI TO KILLING BY THE CATIONIC PROTEINS FROM RABBIT POLYMORPHONUCLEAR LEUCOCYTES: THE EFFECTS OF ALTERATION OF CELLULAR ENERGETICS AND OF VARIOUS IRON COMPOUNDS

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Summary.—Anaerobiosis, various respiratory inhibitors and certain agents altering cellular energetics profoundly affect the staphylocidal action of the cationic proteins from rabbit polymorphonuclear leucocytes. It is suggested that sensitivity to these proteins depends on the structure of the cell membrane as influenced by (1) the oxidation level of the cytochrome chain and (2) its energized state. Agents such as amytal and rotenone, which cause a block at the beginning of the chain and would increase its oxidation level, enhance killing, whereas those causing a block in or at the end of the chain, such as 2-n-heptyl-4-hydroxyquinoline-N-oxide, cyanide and anaerobiosis, which would cause reduction of a part or whole of the chain, prevent killing. Among agents altering the energized state of the membrane, dicyclohexyl-carbodi-imide, an ATPase inhibitor, does not prevent killing, whereas 2,4-dinitro-phenol, carbonylcyanide-trifluoromethoxy-phenylhydrazone and 5-Cl, 3-t-butyl, 2′-Cl, 4′-NO_2-salicylanilide, all uncouplers and ionophores for a specific ion, do prevent killing, although gramicidin, a relatively nonspecific ionophore, does not.

The paper also contains an extension of previous work on the effect of iron and haematin, to include various other iron compounds and haematin derivatives.

PREVIOUS WORK (Gladstone and Walton, 1970; Gladstone, 1973) has shown that iron and haematin prevent the bactericidal action of the cationic proteins from rabbit polymorphonuclear leucocytes. The inhibitory effect of these substances is associated with their ability to precipitate the cationic proteins and originally gave rise to the idea that the cytochromes of the staphylococcal cell membrane may be the sites of action of these proteins. This was also indicated by the studies of Penniall and Zeya (1971) and Penniall, Holbrook and Zeya (1972) on the action of leucocyte cationic proteins on rat liver mitochondria and cytochrome oxidase, and by the demonstration that histones bind to the staphylococcal cell membrane (Hibbitt and Benians, 1972). The suggestion was supported by studies of the effect of cultural conditions on the susceptibility of whole staphylococci to the cationic proteins, and by studies of the action of the proteins on the staphylococcal cell membrane. Staphylococci grown under conditions of repression by glucose or other fermentable carbohydrates are diverted to a fermentative mode of metabolism and are resistant to killing (Gladstone, Walton and Kay, 1974). Staphylococci grown under anaerobic conditions are also resistant to killing (Gladstone and Kay, unpublished observations). An investigation of the effect of the cationic proteins on the NADH oxidase activity of the staphylococcal cell membrane suggested that a functional cytochrome chain is necessary for the binding and subsequent action of these proteins (Walton and Gladstone, 1975).

Work with other bacteria has also implicated the cell membrane in the

bactericidal action of cationic agents. Rosenthal and Buchanan (1974) demonstrated that various cationic agents including histone and β-lysin stimulate the membrane ATPase of *Bacillus subtilis*. In *Bacillus megaterium* Gooch and Donaldson (1974) have shown that β-lysin causes the membranes to fragment and lose their unit structure.

In this paper the effects on the staphylocidal action of the cationic proteins of (1) addition of various iron and haematin derivatives and (2) alteration in the respiratory and energetic capacities of the bacteria are described.

MATERIALS AND METHODS

Staphylococci.—Most of the work was carried out with *Staphylococcus aureus*, strain P66. Other strains used included *Staph. aureus*, 18Z, and *Staph. epidermidis*, Dubos "Air" (for references see Gladstone and Walton, 1971). Organisms were grown overnight in a T-tube in Brain Heart Infusion broth (BHI), and prepared for use as previously described (Gladstone *et al.*, 1974).

Materials.—Ascorbic acid, dithiothreitol (DTT), thioglycollic acid, 2-mercaptoethanol, L-cysteine-HCl, malonic acid, potassium cyanide and 2,4-dinitrophenol (DNP) were obtained from British Drug Houses. Horse ferritin and cytochrome *c* were obtained from Dr W. E. van Heyningen. All were dissolved in 0·067 mol/l phosphate buffer to give a final pH of 7·4. "Haemoglobin" (containing up to 75% methaemoglobin), haematin, glutathione, amytal, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), rotenone, dicyclohexylcarbodi-imide (DCCD), valinomycin and gramicidin were obtained from Sigma Chemical Corp. Carbonyl-cynanide-trifluoromethoxy-phenylhydrazone (FCCP) was obtained from Boehringer Corp. Myoglobin was obtained from Koch-Light Chemicals and 5-Cl,3-t-butyl,2′-Cl,4′-NO_2-salicylanilide (S13) from Lifebuoy (Williamson and Metcalf, 1967). Deuterohaematin, haematohaematin, haematoporphyrin, mesohaematin and urohaematin were obtained from Dr W. E. van Heyningen (1951). Stock solutions were made as follows: haemoglobin (50 mg/ml), myoglobin (12·5 mg/ml), glutathione (50 mmol/l) all in 0·067 mol/l phosphate buffer, pH 7·4; haematin and derivatives, 2 mg/ml in 0·1 mol/l NaOH; amytal, 0·25 mol/l in 0·5 mol/l NaOH; HOQNO, 50 μg/ml in 0·01 mol/l NaOH; rotenone, 2·5 mmol/l in ethanol; valinomycin and gramicidin, 50 μg/ml in ethanol; DCCD, 50 mmol/l in ethanol; FCCP and S13, 0·25 mmol/l in ethanol.

Apolactoferrin and Fe-saturated lactoferrin were obtained as previously described (Gladstone and Walton, 1971).

Cationic proteins (granular extract: GE) from the lysosomes of rabbit polymorphonuclear leucocytes were prepared as previously described (Gladstone *et al.*, 1974). Treatment of GE with dithiothreitol was carried out as follows: 0·4 ml $(NH_4)_2SO_4$ slurry of GE was dialysed against PBS for 5 h at 4°. The insoluble residue was removed by centrifugation and discarded. The supernatant, which contained the cationic proteins, was divided in half. One half was treated with an equal volume of 0·125 mol/l dithiothreitol, dissolved in 0·067 mol/l phosphate buffer, pH 7·4, for 15 min at 37°. The other half was similarly treated using phosphate buffer alone. Dialysis against PBS was then carried out for a further 15 h.

All solutions were sterilized by filtration through a membrane filter prior to use in the test killing system.

Test of susceptibility to killing by GE.—Susceptibility to GE was tested as previously described (Gladstone *et al.*, 1974) using the chemically defined medium of either Kloos and Pattee (1965) or Gladstone (1937), usually without glucose and vitamins. These small variations in the test medium made no difference to the degree of killing. The amino acid media were prepared in a five-fold concentration, at pH 7·4, and diluted before use with 0·067 mol/l phosphate buffer, pH 7·4. Additions to the test medium were made in minimum volumes of solution, adjusting the volume of diluting buffer appropriately. When ethanolic solutions were added, the lowest possible concentrations of ethanol were used, and since ethanol alone enhances killing by GE, the effect of any reagent added in ethanolic solution was always assessed by comparison with a test system containing GE and the appropriate concentration of ethanol alone. If additions of alkaline solutions were made the pH of the test medium was adjusted back to 7·4. Tests under anaerobic conditions were carried out in the vessel shown in Fig. 1 in an atmosphere of H_2–5% CO_2, and in the presence of either 20 mmol/l ascorbate or 2 mmol/l thioglycollate as reducing agent. The redox potential was monitored throughout the experiment using a micro-combination platinum electrode obtained from Electronic Instruments Ltd., Surrey.

Viable counts were carried out as previously described on samples taken from zero time to 4 h. Under aerobic conditions the dose of GE added (final concentration 40–80 μg protein/ml) was usually sufficient to give a reduction in viable count to 0·01% over 4 h (10^6 to 10^2 cfu/

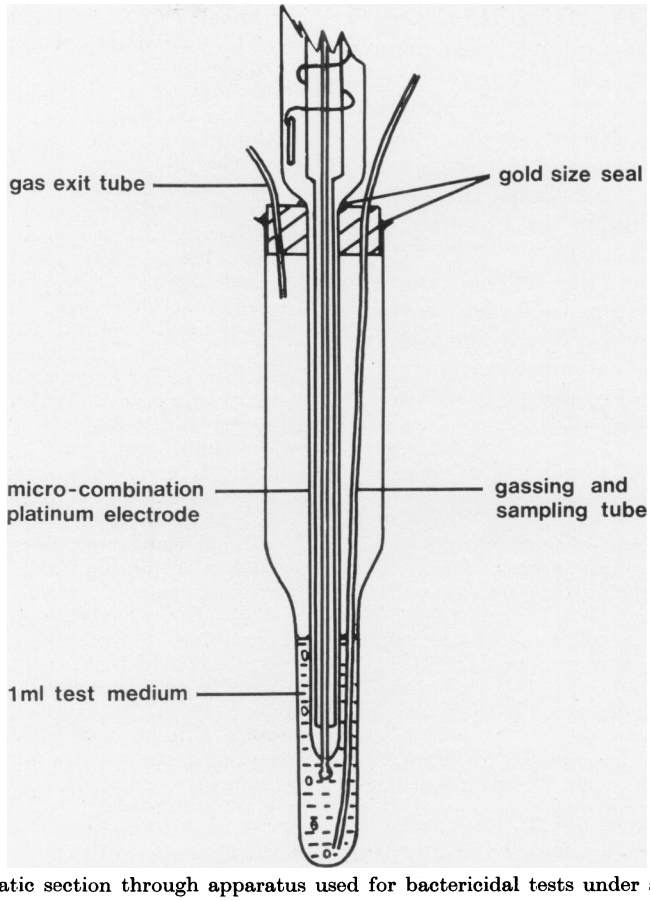

FIG. 1.—Diagrammatic section through apparatus used for bactericidal tests under anaerobic conditions.

ml), but sometimes the reduction in viable count was to only 0·5%.

Measurement of oxygen uptake by whole staphylococci.—Oxygen uptake was measured using a Clark type oxygen electrode supplied by Rank Brothers, Cambridge, attached to a Servoscribe recorder. The incubation vessel contained 0·25 ml air-saturated Kloos and Pattee medium at 37°, with 2×10^8 organisms/ml.

RESULTS

The effect of iron-containing compounds and some iron-free derivatives on the killing of staphylococci by cationic proteins

Table I summarizes the effects of iron-containing compounds and iron-free derivatives on the killing of staphylococci by GE. The complete inhibition of killing which may occur in the presence of Fe (>0·5 mmol/l) and haematin (0·02 mg/ml ≡ 0·03 mmol/l Fe), and the precipitation of GE caused by these compounds, have been previously reported (Gladstone and Walton, 1970; Gladstone, 1973). The latter report also stated that haemoglobin (which was tested in concentrations up to 4 mg/ml) did not have these actions on GE. However, further study has shown that if high enough concentrations of haemoglobin are used (8 mg/ml ≡0·48 mmol/l), both inhibition of killing and precipitation of GE may occur. Myoglobin, ferritin and cytochrome *c*, in concentrations containing similar amounts of iron, also effectively inhibit killing, but cytochrome *c* does not appear to precipitate GE like the others. Of the haematin derivatives, haematohaematin is similar to haematin in the concentration

TABLE I.—*Effect of Iron-Containing Compounds and Some Iron-Free Derivatives on the Killing of *Staphylococci by Cationic Proteins from Rabbit Leucocytes*

Compound	Toxicity	Precipitation of GE	Effect on killing
$FeSO_4$	None	+	0·5–2 mmol/l inhibits. Degree depends on strain (Gladstone and Walton, 1970)
Haematin	None	+	Complete inhibition ⩾0·02 mg/ml (≡ 0·03 mmol/l Fe)
Haematohaematin	None	+	Complete inhibition ⩾ 0·02 mg/ml
Haematoporphyrin	None	+	Complete inhibition ⩾ 0·02 mg/ml
Urohaematin	None	+	0·16 mg/ml (≡ 0·24 mmol/l Fe) inhibit killing markedly (100-fold). 0·02 mg/ml no effect
Haemoglobin	None Stimulates growth	+	8 mg/ml (≡ 0·48 mmol/l Fe) inhibits completely. Lower concentrations very little effect
Myoglobin	Slightly toxic on prolonged incubation	+	32 mg/ml (≡ 0·48 mmol/l Fe) inhibits completely. ⩽ 16 mg/ml no effect
Ferritin	None Stimulates growth	+	1·3 mg/ml (≡ 4·6 mmol/l Fe) inhibits completely. < 0·32 mg/ml no effect
Cytochrome *c*	None Stimulates growth	−	8 mg/ml (≡ 0·57 mmol/l Fe) inhibits completely. ⩾ 4 mg/ml inhibits slightly (4-fold or less)
Fe-lactoferrin	At 0·2 mg/ml non-toxic and may stimulate growth. At 1 mg/ml toxic	?	0·2 mg/ml (≡ 0·005 mmol/l Fe) inhibits. Degree depends on strain (Dubos, 300-fold; 18Z, complete)
Apolactoferrin		?	None (0·2 mg/ml)

* *Staph. aureus*, strain P66, unless otherwise stated.

(0·02 mg/ml≡ 0·03 mmol/l Fe) at which it is effective in preventing killing and in its precipitation of GE. However, its iron-free derivative, haematoporphyrin, also precipitates GE and prevents killing at this low concentration, whereas urohaematin only does so at the higher concentration of 0·16 mg/ml (≡0·24 mmol/l Fe). Neither haematin, nor these 3 derivatives, have any effect on the uptake of oxygen by *Staph. aureus* (P66). Deuterohaematin and mesohaematin are both very toxic to *Staph. aureus* and could not, therefore, be tested in the presence of GE.

The addition of haematin to the system after killing has started, prevents, and in some cases appears to reverse, the killing process (Fig. 2).

The studies on lactoferrin have been previously reported (Gladstone, 1973). Fe-lactoferrin is another iron-containing molecule which is effective at low concentrations (0·2 mg/ml ≡ *0·005 mmol/l Fe) in inhibiting killing by GE. The iron-free derivative has no effect on killing.

From these results it appears that iron prevents killing by combining with and precipitating GE. Haemoglobin, myoglobin and ferritin may also act in this way by virtue of their iron content. Similarly, cytochrome *c*, although it fails to precipitate GE, may inhibit killing by combining with GE to form a soluble complex. Fe-lactoferrin also seems to prevent killing because of its iron content, since apolactoferrin is inactive. Haematin and its derivatives may fall into a different category. Their iron content may not be relevant, since a porphyrin, as well as various haematins, all prevent killing and all precipitate GE.

The effect of anaerobiosis and reducing agents

The following reducing agents were tested under aerobic conditions for their toxicity to *Staph. aureus* (P66) and for their effect on killing of this organism by

*Erroneously given as 0·01 mmol/l Fe in Gladstone (1973).

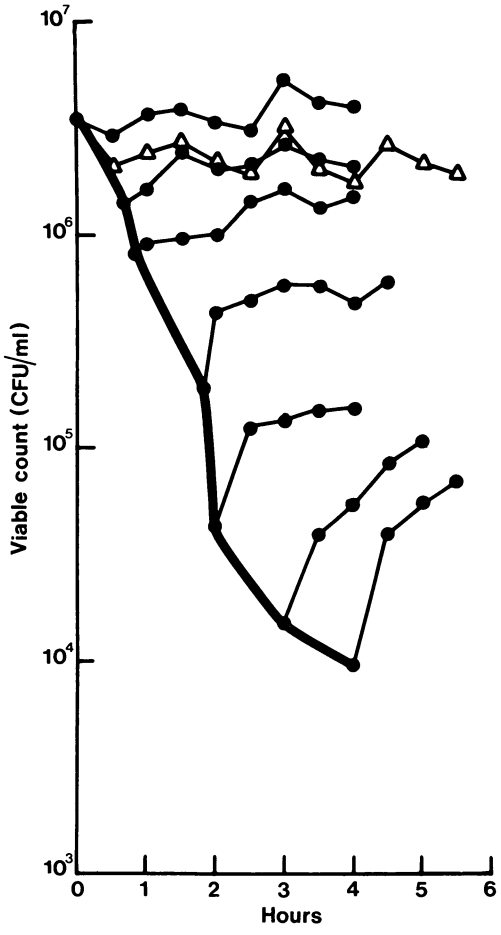

FIG. 2.—Sparing effect of haematin added during the course of killing of *Staph. aureus* (P66) by GE. Thick line represents killing curve with GE alone; △———△, no additions; ●———●, *plus* GE *and* haematin (0·16 mg/ml).

GE: glutathione, cysteine, mercaptoethanol, thioglycollate, dithiothreitol and ascorbate. As for most of the experiments described in this paper, 0·25 ml test medium was contained in 3-ml capped plastic tubes. The tubes were kept stagnant, at 37°, and the area of medium exposed to the air was approximately 0·75 cm^2. Glutathione and cysteine, tested in concentrations up to 10 mmol/l are the most toxic causing a fall in viable count of 10^6 to 10^3 organisms/ml over 4 h. They appear to have no effect on killing by GE. Mercaptoethanol at the same concentrations is less toxic. At 10 mmol/l it only reduces the viable count from 10^6 to 10^5 organisms/ml over 4 h, and at this concentration it also slightly inhibits killing by GE, the viable count after 4 h being 10^4 organisms/ml in its presence and 10^2 organisms/ml with GE alone. Thioglycollate at 0·4–2 mmol/l has no significant effect on viability, but at 10 mmol/l it is toxic, causing a fall in viable count of 10^6 to 10^4 organisms/ml over 4 h. It does not affect killing by GE. Ascorbate is anomalous in showing an inverse relationship between concentration and toxicity: at 10 or 20 mmol/l it has no effect on viability, but at 2 or 5 mmol/l is slightly toxic, lowering the viable count from 10^6 to 5×10^4 organisms/ml over 4 h. It has no inhibitory action on killing by GE.

Figure 3 shows that dithiothreitol behaves differently from the other reducing agents in the presence of GE. Like ascorbate it has no effect on viability at higher concentrations (5, 10 mmol/l), but is toxic at lower concentrations (1, 2·5 mmol/l). However, in the presence of GE, there is a steadily increasing inhibition of killing as the concentration of dithiothreitol rises, there being little or no inhibition at 1 mmol/l. Sometimes, as shown in the figure, growth may occur with 5 or 10 mmol/l dithiothreitol in the presence of GE. On other occasions there is no growth, but killing is completely or partially relieved. Pre-treatment of GE with dithiothreitol, followed by dialysis to remove the reducing agent, causes a complete loss of killing activity.

The redox potential (E_h) of stagnant test media, with and without reducing agents, was measured under the same conditions as the killing test except that 0·5 ml medium was used. The measurements show that the occurrence of killing by GE is directly related to the E_h of the medium. Negative redox potentials are obtained over the course of a 4 h incubation only in the presence of 2·5, 5 and 10 mmol/l dithiothreitol (— 0·064, — 0·109, — 0·144 V respectively), all of

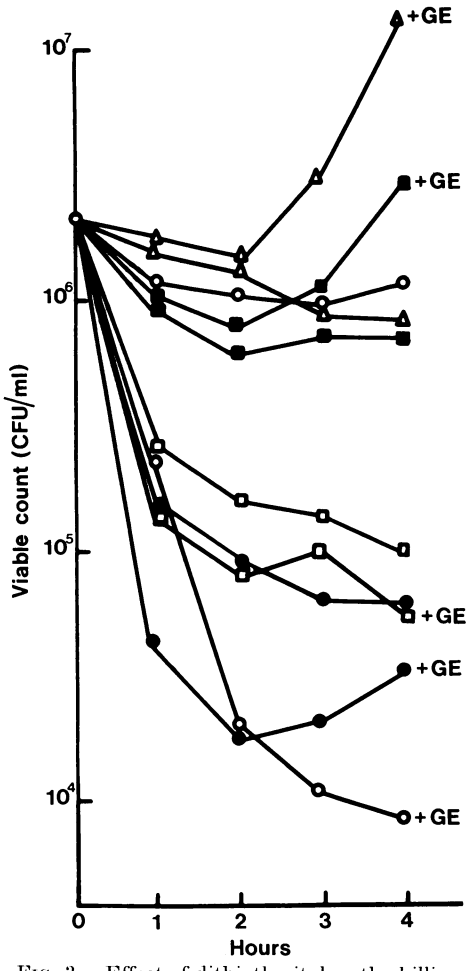

FIG. 3.—Effect of dithiothreitol on the killing of *Staph. aureus* (P66) by GE. ○———○, no dithiothreitol (DTT); ●———●, *plus* 1 mmol/l DTT; □———□, *plus* 2·5 mmol/l DTT; ■———■, *plus* 5 mmol/l DTT; △———△, *plus* 10 mmol/l DTT. GE present where indicated. In the presence of GE, dithiothreitol at high concentrations may promote growth, as shown in this figure.

which inhibit killing by GE. A negative E_h is also obtained in the first hour of incubation in the presence of 10 mmol/l mercaptoethanol (— 0·009 V), which slightly inhibits killing by GE.

Experiments under anaerobic conditions were carried out using either ascorbate or thioglycollate as reducing agents since these have very little toxicity and no effect on killing by GE. Fig. 4 shows representative experiments. Killing is completely prevented by carrying out the incubation with GE under anaerobic conditions, when the E_h is below — 0·2 V. However, restoration of aerobic conditions, by bubbling air–5% CO_2 through the test medium after 4 h, does not restore susceptibility of the organism to GE (Fig. 4*b*).

The effect of respiratory inhibitors

Table II summarizes the effect of various respiratory inhibitors on the killing of *Staph. aureus* (P66) by GE. With the exception of malonate, a correlation can be drawn between the site of action of the inhibitor and its effect on killing. Amytal and rotenone block electron transfer early in the cytochrome chain, and enhance killing by GE. HOQNO blocks electron transfer later in the chain. It enhances killing at low concentrations, and inhibits it at higher concentrations. Cyanide, which inhibits cytochrome oxidase, thereby blocking electron transfer at the end of the chain, has only an inhibitory effect on killing.

Malonate inhibits succinic dehydrogenase and other dehydrogenase sites near the beginning of the cytochrome chain, yet, unlike amytal and rotenone, it inhibits killing. However, studies with membrane preparations have shown that it has a further action. The NADH oxidase activity of staphylococcal membrane preparations is inhibited by GE (Walton and Gladstone, 1975), but this inhibition is itself relieved by malonate (unpublished observations).

Experiments using amytal and cyanide together have yielded useful information (Fig. 5). Both reagents may be added to the killing system after an incubation of 1 h and still exert their characteristic effects (Fig. 5*a* and *b*). If amytal is added at zero time, followed by cyanide at 1 h (Fig. 5*c*), killing is stopped. In the reverse experiment (Fig. 5*d*), when amytal is added to a system in which killing is already prevented by cyanide, no killing is initiated. Cyanide, therefore, seems to

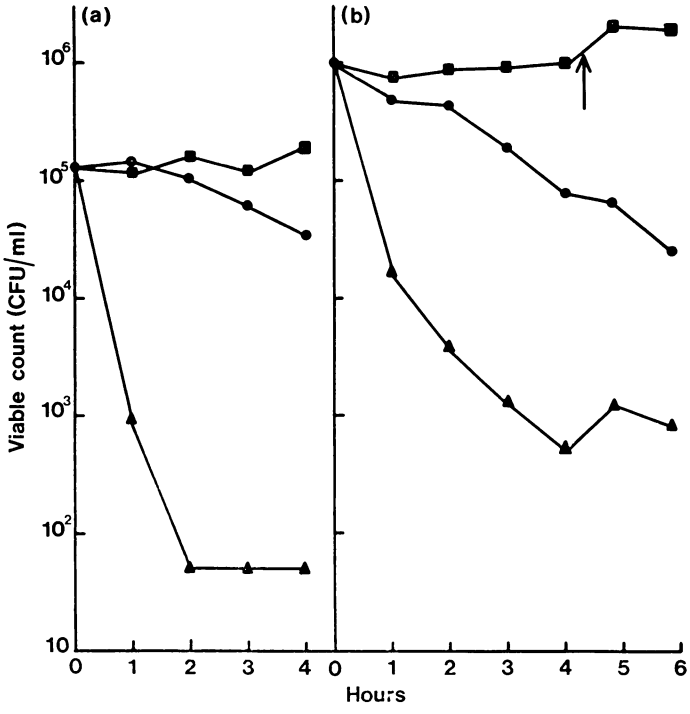

FIG. 4.—Effect of incubation under anaerobic conditions on the killing of *Staph. aureus* (P66) by GE. (a) Incubations carried out in the presence of 20 mmol/l ascorbate. Average E_h in anaerobic incubation = − 0·206V. (b) Incubations carried out in the presence of 2 mmol/l thioglycollate. Average E_h in anaerobic incubation = − 0·343V. In this experiment the anaerobic incubation was made aerobic at 4½ h by bubbling air–CO_2 through the medium (↑), when the E_h became positive. ●———●, aerobic control (no GE, air–CO_2); ■———■, anaerobic (H_2–CO_2) *plus* GE; ▲———▲, aerobic (air–CO_2) *plus* GE.

over-ride any effect of amytal in the system, which suggests that amytal does not completely block the cytochrome chain at the beginning, so that cyanide can still block it effectively at the end.

The effect of inhibition of energy transfer and of uncoupling or inhibition of oxidative phosphorylation

Table III summarizes the effect of various agents affecting cellular energetics on the killing of *Staph. aureus* (P66) by GE. The ATPase inhibitor, DCCD, enhances the action of GE at all concentrations at which it was tested. The other agents, although they are all ionophores, do not all affect killing in the same way. DNP, FCCP and S13, all e'ectrogenic proton conductors which uncouple oxidative phosphorylation, inhibit killing by GE. Valinomycin, which is similar in that it conducts electrogenic movement of a specific ion (K^+), but which may or may not uncouple oxidative phosphorylation, also inhibits killing by GE, though not completely. Gramicidin, which conducts several monovalent cations and uncouples oxidative phosphorylation, enhances killing by GE.

DISCUSSION

The observations described in this paper give some indication of the mechanism of action of the cationic proteins on staphylococci.

The studies under anaerobic conditions and in the presence of reducing agents may be interpreted in two ways: (1) staphylococci grown under aerobic conditions but placed in highly reducing or

TABLE II.—*Effect of Respiratory Inhibitors on the Killing of* Staph. aureus (*P66*) *by Cationic Proteins from Rabbit Leucocytes*

Inhibitor	Site of action	Toxicity	Effect on killing
Malonate	Inhibits succinic- and other dehydrogenases. Relieves inhibition of NADH oxidase by GE which occurs in staph. membrane preparations. (Walton and Gladstone, unpublished observations)	None up to 0·1 mol/l	⩾ 0·05 mol/l inhibits 300-fold, 0·1 mol/l almost completely (1000-fold)
Amytal	Inhibits electron transfer in region of NAD- and succinate-linked flavoprotein. May also inhibit energy transferase reactions at this site. (Taber and Morrison, 1964)	None up to 10 mmol/l	1–10 mmol/l enhance up to 200-fold
Rotenone	Inhibits electron transfer at a site early in the chain between NAD and cytochrome *b*	None using saturated solution (0·05 mmol/l)	Saturated solutions enhance slightly (up to 30-fold)
HOQNO	Inhibits electron transfer between cytochrome *b* and terminal portion of chain (Taber and Morrison, 1964)	None up to 0·15 mmol/l	Low concentrations (0·01–0·1 μmol/l) enhance up to 8-fold. 1 μmol/l inhibits 0–10 fold. ⩾ 10 μmol/l inhibits completely
Cyanide	Inhibits cytochrome oxidase (cytochrome *o* in staphs.)	None up to 40 mmol/l	5 mmol/l inhibits up to 10-fold, 20 mmol/l inhibits completely

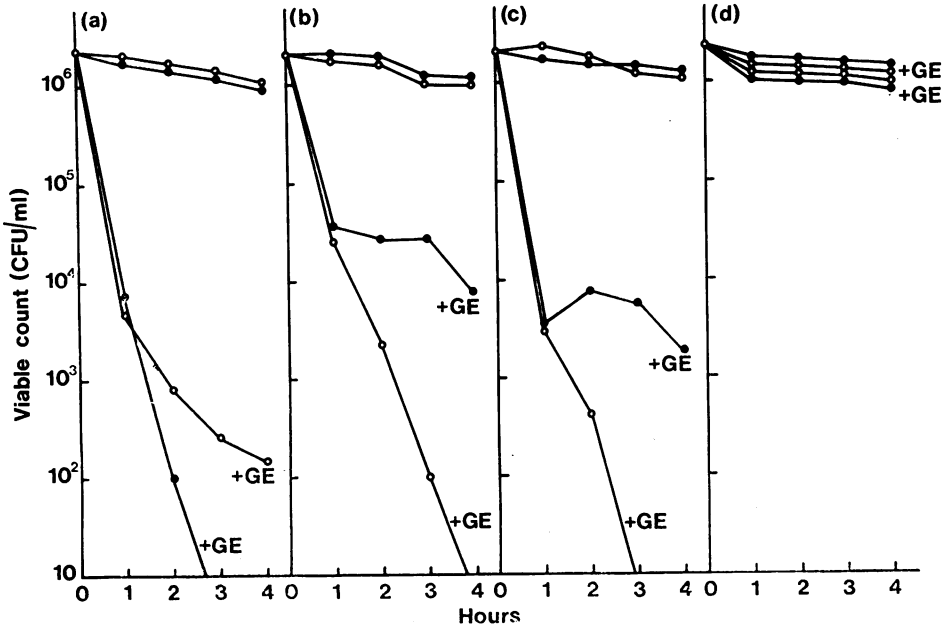

FIG. 5.—Effects of amytal and cyanide on the killing of *Staph. aureus* (P66) by GE. (a) Addition of amytal during the experiment. ○———○, no amytal; ●———●, 1·1 mmol/l amytal added at 1 h. (b) Addition of cyanide during the experiment. ○———○, no cyanide; ●———●, 40 mmol/l cyanide added at 1 h. (c) Addition of cyanide during the experiment in the presence of amytal. ○———○, *plus* 1·1 mmol/l amytal; ●———●, *plus* 1·1 mmol/l amytal, 40 mmol/l cyanide added at 1 h. (d) Addition of amytal during the experiment in the presence of cyanide. ○———○, *plus* 40 mmol/l cyanide; ●———● *plus* 40 mmol/l cyanide, 1·1 mmol/l amytal added at 1 h. GE present where indicated.

TABLE III.—*Effect of Inhibition of Energy Transfer and of Uncoupling or Inhibition of Oxidative Phosphorylation on the Killing of* Staph. aureus (*P*66) *by Cationic Proteins from Rabbit Leucocytes*

Inhibitor	Site of action	Toxicity	Effect on killing
DCCD	ATPase inhibitor. Reacts covalently with a protein component of the membrane	0·5 mmol/l not toxic. 1–2 mmol/l increasingly toxic	All concentrations tested (0·5–2 mmol/l) enhance, up to 1000-fold
DNP	Ionophores. Specifically conduct protons across membranes. Uncouple oxidative phosphorylation. Proton movement electrogenic	None up to 10 mmol/l	⩾2·5 mmol/l inhibits 10-fold, 5–10 mmol/l inhibits completely
FCCP		None up to 10 μmol/l	⩾2·5 μmol/l inhibits up to 5-fold, 5–10 μmol/l almost completely (1000-fold)
S13		Slight from 2·5–10 μmol/l	2·5–10 μmol/l inhibits 10–300-fold. Not as effective as FCCP
Valinomycin	Ionophore. Specifically conducts potassium ions across membranes. Does not always uncouple oxidative phosphorylation. Potassium ion movement electrogenic	0·5 μg/ml very slightly toxic. 1–2 μg/ml toxic	0·5–2 μg/ml inhibits incompletely (up to 100-fold)
Gramicidin	Ionophore. Relatively non-specific for monovalent cations. Conducts protons, sodium and potassium ions. Uncouples oxidative phosphorylation	0·5 μg/ml very slightly toxic. 1–2 μg/ml increasingly toxic	0·5–2 μg/ml enhances up to 150-fold

anaerobic conditions carry out a fermentative mode of metabolism and do not, therefore, have the functional cytochrome chain which has been suggested to be necessary for the action of GE (Walton and Gladstone, 1975); (2) GE itself may be reduced to an inactive form under highly reducing or anaerobic conditions. This interpretation is supported by the demonstration that GE pre-treated with dithiothreitol loses activity, and by the finding that it is not possible to initiate killing on restoration of aerobic conditions to an anaerobic incubation medium containing GE. The mode of metabolism of the staphylococci under these conditions would be immaterial.

The studies with respiratory inhibitors suggest that the level of oxidation of the cytochrome chain is an important factor in allowing killing by GE. The results are consistent with the idea that inhibitors causing a block at the beginning of the cytochrome chain (amytal, rotenone), so that the chain becomes more oxidized, enhance killing, and inhibitors causing a block in or at the end of the chain (CN, HOQNO), so that a part or the whole becomes more reduced, prevent killing. This could indicate a simple relationship between the structure of the bacterial membrane, as determined by the oxidation level of the cytochrome chain, and either the quantity of GE which may bind, or the changes in the membrane consequent on binding.

In whole mitochondria, small-amplitude swelling–contraction cycles can be measured as changes in optical density of a suspension. These changes are active and coupled to the respiratory chain. Larger amplitude swelling–contraction cycles can be induced by respiratory chain inhibitors (Lehninger, 1964). Hackenbrock (1966) has demonstrated reversible changes in the conformation of the inner membrane of mitochondria accompanying changes in metabolic states. Conformational alterations are also seen in the presence of the respiratory inhibitors amytal, antimycin and cyanide (Hackenbrock, 1968). At a higher level of organization Hackenbrock (1973) has shown that changes in the activity of the respiratory chain parallel large structural transformations in the spatial orientation of particles on

the inner electron transport membrane. The response of fluorescent probes such as anthroylstearic acid, which are sensitive to the oxidation/reduction state of the respiratory carriers, also indicates that changes in membrane conformation may occur with respiratory activity (Chance, 1972).

The studies with agents altering cellular energetics are more difficult to interpret. They do not indicate a simple relationship between the energetic state of the cell and susceptibility to GE. Inhibition by DCCD of ATPase, the coupling device which mediates the energy transfer reactions leading to ATP synthesis, does not prevent killing. However, ionophores mediating electrogenic movement of a specific ion (DNP, FCCP, S13) prevent killing by GE, whereas gramicidin, which conducts several monovalent cations, enhances killing by GE. It seems that a discrete change in the energy-conserving properties of the membrane, such as leakiness to specific ions, may interfere with the action of GE, whereas a more profound change, such as reaction with DCCD or non-specific leakiness to cations does not.

These observations are consistent with the suggestion that there is a relationship between the structure of the bacterial membrane, in this case influenced by the energetic state of the cell, and the action of GE on the cell. The large swelling-contraction cycles of mitochondria are not only induced by respiratory inhibitors, but also by inhibitors and uncouplers of oxidative phosphorylation (Lehninger, 1964). Hackenbrock (1968) has demonstrated that DNP inhibits changes in the conformation of the inner membrane of mitochondria. At a higher level of organization the fluorescent probes ANS (1-anilino-8-naphthalene sulphonate) and auramine-O (Azzi, 1969; Harold, 1972), and ACMA (9-amino-6-chloro-2-methoxyacridine, van Thienen and Postma, 1973; Nieuwenhuis *et al.*, 1973), which respond to the energized state of the membrane, have been used in both sub-mitochondrial particles and in bacterial membrane particles or vesicles. They indicate that alterations similar to those occurring with differences in oxidation/reduction state of the membrane may occur with changes in the energized state.

The studies of Johnson and coworkers (Schwartz, Johnson and Starbuck, 1966; Johnson, Safer and Schwartz, 1966; Johnson, Mauritzen *et al.*, 1967; Johnson, Oro and Schwartz, 1969; Johnson, Goldstein and Schwartz, 1973) on the effects of histones on isolated heart and liver mitochondria in the presence of various inhibitors of electron transfer, oxidative phosphorylation and energy transfer give similar but not identical results to those described in this paper. The effects of the basic proteins on mitochondria include the stimulation of oxygen consumption, ATPase activity, potassium ion efflux and swelling, and they are dependent on the availability of metabolic energy. When the energy is supplied by electron transport these effects are prevented by all electron transport inhibitors tested, irrespective of the position of the block which they cause in the respiratory chain. They are also prevented by the action of uncoupling agents such as DNP. When the energy source is ATP, the effects of the basic proteins are prevented by energy transfer inhibitors such as oligomycin, which is similar to DCCD in acting on ATPase. Membrane-stabilizing agents such as local anaesthetics or chlorpromazine, which have little or no effect on electron transport or oxidative phosphorylation, also prevent the effects of the basic proteins.

There are other studies which indicate that two highly specific antibacterial systems also depend on bacterial oxidative energy metabolism. The action of colicins on various bacteria can be prevented by anaerobiosis, respiratory inhibitors and uncouplers and inhibitors of oxidative phosphorylation (Reeves, 1972; de Graaf, 1973). Similarly, Griffiths (1974) has demonstrated that the killing of *Pasteurella septica* by antibody

and complement can be prevented by inhibiting aerobic metabolism with cyanide or anaerobiosis, or by uncoupling oxidative phosphorylation with DNP or CCCP (carbonylcyanide *m*-chlorophenylhydrazone).

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