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Electronmicroscopy of the anti-bacterial agent produced by *Escherichia coli* 15

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SUMMARY

Some properties of the phage ψ of *Escherichia coli* 15, as revealed by electronmicroscopy of an u.v.-induced lysate, are described. The differences between this defective phage and a bacteriocine and the type of interaction between this phage and *E. coli* 15 are discussed.

INTRODUCTION

When it was first observed that the strain 15 of *Escherichia coli*, after u.v.-irradiation and further incubation, would eventually lyse and release an anti-bacterial agent, this agent was considered to be a 'colicine' (Mukai, 1960; Ryan, Fried & Mukai, 1955). The possibility that, in addition, a defective phage was produced, was left open. But, since in an u.v.-induced lysate of this strain all anti-bacterial activity can be spun down by applying centrifugal forces of only 20,000 g for 2 hr (Mukai, 1960) and since those colicinogenic strains, which do lyse after u.v.-irradiation, were found to be also lysogenic (Kellenberger & Kellenberger, 1956), we concluded (Mennigmann, 1964*b*) that the anti-bacterial agent must be a large particle, probably a phage. In this communication we wish to verify these conclusions by presenting some electronmicroscopical pictures of such lysates, which show a defective phage to be present. Thus, regarding the antibacterial agent produced by *E. coli* 15, we rather favour the opposite of the above picture: the anti-bacterial agent is a defective phage and the possibility of the additional presence of a colicine has to be left open.

METHODS

The bacterial strain *Escherichia coli* 15 T$^-$—obtained through the courtesy of Professor O. Maaløe—was grown in a minimal medium (Maaløe & Hanawalt, 1961) enriched with 1·5% Difco casamino acids and 2×10^{-5} M thymine. A logarithmically growing culture (OD578 = 0·15/cm.) was u.v.-irradiated (layer of about 1 mm., 250 ergs/mm.^{2} from an Osram germicidal lamp HNS 12 ofr) and further incubated. At the time of maximum lysis the culture was shaken with 1/10 volume chloroform for 15 sec. and the bacterial debris centrifuged at low speed. From the supernatant specimens were prepared by the phosphotungstate negative staining method and examined under the electronmicroscope 'Siemens Elmiscop I'.

RESULTS

The pictures revealed a phage about which the following can be said. It consists (Pl. 1, fig. 1) of a polyhedric head (about 600 Å in diameter) and a tail (overall length of about 1100 Å, and about 170 Å in diameter). In some cases, the head seems to be empty (Pl. 1, fig. 2). The tail is complex: an outer sheath (Pl. 1, figs 2–4) covers an inner core (Pl. 1, figs. 2, 4). To this is attached a base plate with at least three spikes (Pl. 1, figs. 1, 3 and 5). No fibres could be detected. The sheath appears 'striated' (Pl. 1, figs. 1–4) and is contractile (Pl. 1, figs. 2 and 4). The part of the tail between the head and the sheath is of smaller diameter than the sheath, but bigger than the core and is somewhat reminiscent of the 'neck' of the T-even phages. Thus, this new phage is not much different from *Escherichia coli* phages of the T-even series, except that its head is somewhat smaller. We have named this phage ψ (because the Greek letter psi is—with sufficient imagination—reminiscent of an inner core plus base plate plus three spikes; see Pl. 1, fig. 5).

A surprising feature of the lysate, prepared as described above, was the relatively large number of tails without heads as compared with the number of morphologically 'intact' phages. Even though this treatment does very little, if any, harm to *Escherichia coli* phages of the T-series, it was thought possible that some kind of destruction might have occurred when shaking the crude lysate with chloroform. But omitting this purification step did not result in fewer tails as compared with the number of 'intact' phages. Thus, it can be concluded either that the phage is so fragile that it falls apart when the bacterial cell lyses, or that the 'assembly machinery' does not work properly, or that the cells predominantly produce tails. In favour of the latter possibility is the fact that one does not find as many heads as one can see tails (assuming that the heads are not preferentially obscured by the large amount of cell debris still present after low-speed centrifugation).

In addition to the phages or phage-related structures there are at least two more types of particles with a defined shape, which have not been found in T4- or λ-lysates as obtained from other strains of *Escherichia coli* and whose nature so far is unknown: round cyst-like particles much smaller than the phage head and four-lobed structures (Pl. 1, fig. 6).

DISCUSSION

It was found that by u.v.-irradiation (Mukai, 1960; Ryan *et al.* 1955) and by thymine deprivation (Luzzati & Chevallier, 1964; Mennigmann, 1964*a*; Sicard & Devoret, 1962) the bacterial strain *Escherichia coli* 15 can be induced to lyse, and that it concomitantly produces an anti-bacterial agent—presumably a colicine—active against the same bacterial strain (Mukai, 1960; Ryan *et al.* 1955). From the sedimentability at 20,000 *g* (Mukai, 1960) it was concluded (Mennigmann, 1964*b*) that the agent must be larger than other known true colicines. Because it was found (Kellenberger & Kellenberger, 1956) that, whenever a colicinogenic strain lyses after u.v.-irradiation, phages are also produced, we assumed this also to be the case (Mennigmann, 1964*b*) with the presumably colicinogenic strain *E. coli* 15 (Mukai, 1960; Ryan *et al.* 1955). This assumption was verified by the finding reported in this communication at least to the extent that phages (and parts of them) are produced. There is no evidence for the presence, in addition to the particulate agent, of an ordinary colicine. The nature of the other constituents of the

lysate (Pl. 1, fig. 6) will have to be elucidated. Thus, since the lysate can be freed from the anti-bacterial activity by the same centrifugal forces which effectively sediment phages, we conclude that the active agent in lysates from *E. coli* 15 is the defective phage found in the electronmicroscopical pictures. The observation (Mukai, 1960) that the anti-bacterial agent is sensitive to the action of commercial trypsin was thought to be in favour of the agent being a bacteriocine rather than a bacteriophage (Mennigmann, 1964*b*). This argument no longer seems to be valid, since in the meantime Northrop (1964) has reported, that some phages are also sensitive to this enzyme. Thus, the only remaining observation, which was said (Mennigmann, 1964*b*) to argue against the agent being a bacteriophage, is its non-transmissibility. Since this argument only holds true if one excludes defective phages, it is only a very vague one—as one can see from the findings reported in this communication.

In connexion with the fact that *Escherichia coli* 15 is lysogenic for a defective phage, which after induction produces mostly parts of phages, it is interesting to note that the same has been reported for other strains of *E. coli* (Arber & Kellenberger, 1958) and for a strain of *Proteus mirabilis* (Taubeneck, 1963). In the case of virulent phages a number of mutants have been found, which on certain strains of *E. coli* produce complete phages, while on other strains they only produce parts of phages (Epstein *et al.* 1963; Kellenberger & Boy de la Tour, 1963).

Finally, knowing now that the presumed colicine (Ryan *et al.* 1955; Mukai, 1960) in fact seems to be a phage (or part of it), it might be interesting to study more closely (*a*) the observed relationship (Mukai, 1960) between the acquired sensitivity to the other T-phages of *Escherichia coli* and the concomitantly acquired resistance to the phage *ψ*, and (*b*) the type of interaction between this phage and the sensitive strain of *E. coli* 15, which leads to a reversible inhibition of cell division (Mennigmann, 1964*b*). The latter might be of particular interest, since from what has been reported so far it seems to be equally difficult to classify this interaction as 'resistance' (= no adsorption), 'restriction' (= destruction of the injected DNA), or 'immunity' (= prevention of the phenotypic expression of the lytic functions).

The preparation of the excellent electronmicrographs by E. Boy de la Tour and the stimulating discussions with R. W. Kaplan, G. and E. Kellenberger, and R. Thomas are gratefully acknowledged.

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EXPLANATION OF PLATE

Constituents of a crude lysate of *Escherichia coli* 15 T⁻ after u.v.-induction (× 300,000).

Fig. 1. Morphologically 'intact' phage particle.

Fig. 2. Phage particle with an empty head and contracted sheath, thus making visible part of the inner core.

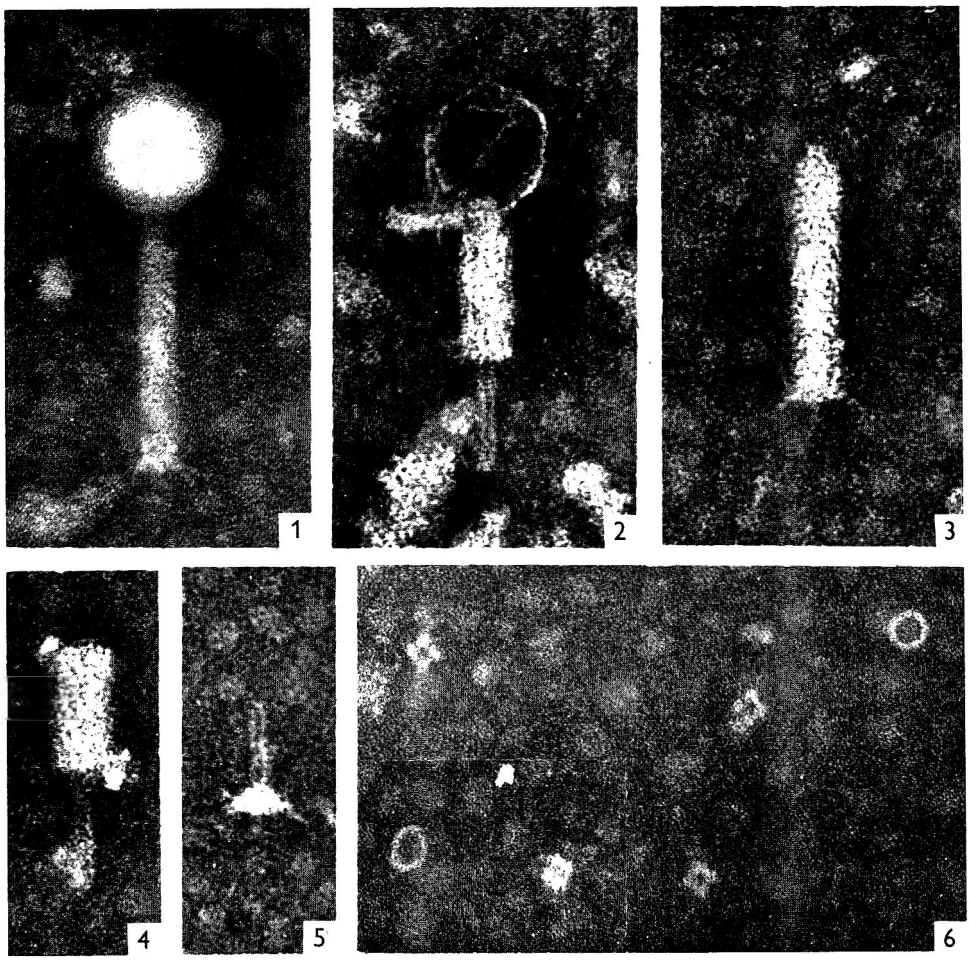
Fig. 3. Phage tail with base plate and spikes. This type of particle is more prevalent than the other phage-related structures.

Fig. 4. Phage tail with contracted sheath, thus making visible part of the inner core.

Fig. 5. Part of the inner core and base plate with spikes.

Fig. 6. Cyst-like particles and four-lobed structures frequently seen in the lysates.

Note added in proof. While this communication was in the press, two other papers have been published which also show that *Escherichia coli* 15 harbours a defective prophage (Endo *et al.* 1965; Sandoval, Reilly & Tandler, 1965).



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Effects of Certain Penicillins on Growth and Cell Division in a Species of *Erwinia*

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SUMMARY

By using growth and division-inhibition at 16 hr as indices of activity, eight penicillins, differing in the side chain, were tested against an *Erwinia* species, and compared with benzylpenicillin. DL-α-Aminobenzylpenicillin, DL-3-chloro-α-aminobenzylpenicillin, D-α-aminobenzylpenicillin, and methicillin were more toxic for growth than was benzylpenicillin. 6-Aminopenicillanic acid and cloxacillin were about equal to benzylpenicillin in toxicity; triphenylmethylpenicillin and 2-ethoxy-1-naphthylpenicillin were much less toxic. All the compounds inhibited division of the bacteria but, at 50% of normal growth, methicillin, cloxacillin and 2-ethoxy-1-naphthylpenicillin resulted in significantly longer organisms than the others. Pantoyl lactone, when present in the medium from the time of inoculation, in all cases decreased the length of the organisms, increased growth (with a highly toxic concentration of penicillin), and decreased the accumulation of keto acids and ultraviolet-absorbing materials in the medium. It was concluded that the side chain of these penicillins is not essential for the inhibition of growth and division of this *Erwinia* sp.; quantitative differences in activity associated with the side chain appear to result from the influence of the side chain on factors such as penetration, or strength of binding at the active site.

INTRODUCTION

The relationship of the structure of the side chain to the activity of various penicillin derivatives against Gram-negative bacteria has not been extensively studied. According to Cooper (1956) the side chain of benzylpenicillin is not important for activity against Gram-positive organisms. However, Russell (1962) observed that 6-(2,6-dimethoxybenzamido)penicillinic acid (dimethoxyphenylpenicillin, Celbenin, methicillin) was by far the least toxic of a series of penicillins for *Escherichia coli*. Boman & Eriksson (1963), using the minimum concentration necessary to produce lysis of *E. coli* K-12 in one generation during log. phase as an index of activity, reported that the side-chain structure markedly influenced activity. They found, in agreement with other workers (Rolinson & Stevens, 1961; Rogers & Mandelstam, 1962), that D-α-aminobenzylpenicillin (D-ampicillin) was considerably more active than benzylpenicillin (penicillin G). The concentration which produced lysis in one generation ('LIOG' value) was about one-tenth as great for D-ampicillin as for benzylpenicillin. D-Ampicillin was about three times as active as L-ampicillin. Boman & Eriksson (1963) found that, in general, aminoalkyl and aminobenzylpenicillins were very active. One exception noted by Cooper (1956)

to the generalization that the side chain was not important for activity toward Gram-positive bacteria was the activity of cephalosporin N, a penicillin which contains α-aminoadipic acid as the side chain (Abraham *et al.* 1953). Boman & Eriksson (1963) also reported that bulky 'non-biological' side chains greatly decreased the antibiotic activity; two such derivatives, methicillin and 3-*p*-chlorophenyl-4-(1,2,5-oxodiazole)-penicillin, were less active than 6-aminopenicillanic acid. The latter compound, lacking a side chain, was one-tenth as potent as benzylpenicillin. By using a different approach, i.e. the ability of a penicillin to inhibit growth and bacterial division when present in the medium from the time of inoculation, we have compared eight penicillins which differ in the side chain, in their activity against an Erwinia.

METHODS

The strain of *Erwinia* sp. used and its maintenance were described previously (Grula, 1960*a*). Tests of antibacterial activity of the penicillins were made in the defined medium of Grula (1960*b*); glucose (autoclaved separately), penicillin, and pantoyl lactone (each filter sterilized) were added just before inoculation. Stock aqueous solutions of the penicillins were always prepared just before use; the correct volume was placed in the medium (buffered at pH 7) less than 20 min. after preparation. Sjöberg & Ekström (see Boman & Eriksson, 1963) found that the half-life of D-ampicillin at pH 7 and 37° was about 100 hr; that of L-ampicillin 15 hr. Since we found DL-ampicillin just as toxic as D-ampicillin, it can be concluded that decomposition of L-ampicillin was not a factor in its observed toxicity.

DL-Pantoyl lactone was bought from Mann Research Laboratories (136 Liberty Street, New York). Stock solutions, adjusted to pH 6·8, could be stored for at least 6 weeks at 4° with no decrease in analytical concentration of lactone.

Five-ml. lots of medium were inoculated with 0·05 ml. of a twice-washed standardized suspension of bacteria from a 24-hr nutrient agar slope. After 16 hr at 25°, with shaking, the cultures were examined for growth and length of organisms. The relative amount of growth was determined by measuring the extinction at 540 mμ (E_{540}) with a Bausch and Lomb Spectronic 20 spectrophotometer. The lengths of organisms were determined as given by Grula & Grula (1962). The average bacterial length given in Table 1 is an arithmetic mean, determined from a size distribution curve, of about 500 bacteria. Bacteria grown without antibiotic under these conditions were uniformly 2–5 μ long at 16 hr. Cultures of very high average bacterial length showed a lesser variability in length; for example, 70% of the culture might consist of filaments > 100 μ long.

After removal of samples for growth and length determinations, cultures were centrifuged and the supernatant fluids analysed for total keto acid by the method of Haidle & Knight (1960). Extinctions at 260 and 280 mμ were measured with a Beckman DU spectrophotometer, with corrections made for the absorption by the penicillin when necessary.

The strain of Erwinia used has never shown evidence of having either a β-lactamase or an amidase for benzylpenicillin. Tests showed that the effects of benzylpenicillin were independent of inoculum size over a 2·5-fold range.

RESULTS

Effects of various penicillins on growth and bacterial division

All the penicillin derivatives tested had some toxicity for the Erwinia strain used (Table 1). In accordance with the results of others (Rolinson & Stevens, 1961; Rogers & Mandelstam, 1962; Boman & Eriksson, 1963) we observed that the aminobenzylpenicillins were more toxic than benzylpenicillin. The difference between DL-α-aminobenzylpenicillin (D:L = 6:4) and the pure D-isomer were not such as to indicate a 3-fold higher activity of the pure D-compound, as reported by Boman & Eriksson (1963).

The only other compound as toxic as the aminobenzylpenicillins was methicillin. This result was in contrast to the results of Boman & Eriksson (1963), who found that methicillin possessed almost no lytic activity for *Escherichia coli*, and to the results of Russell (1962), who used inhibition of growth in nutrient broth and spheroplast formation as indices of activity. Two compounds (triphenylmethylpenicillin, 2-ethoxy-1-naphthylpenicillin), neither of which was tested by Boman & Eriksson, inhibited growth only at concentrations at least three times greater than inhibitory concentrations of benzylpenicillin.

All the derivatives tested inhibited bacterial division (Table 1), resulting in the formation of filaments. Some compounds induced the formation of very long filaments at a concentration which allowed relatively good growth. With other compounds, the average bacterial length was not very great even at concentrations rather inhibitory of growth. The basis of this partial independence of the growth-inhibitory and division-inhibitory properties of these penicillins is not known: it might indicate possible multiple sites of action.

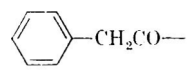
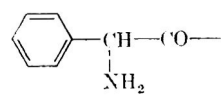
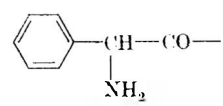
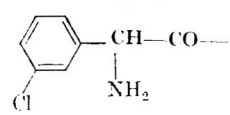
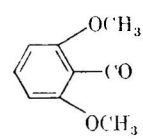
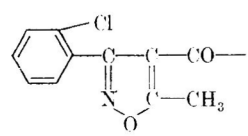
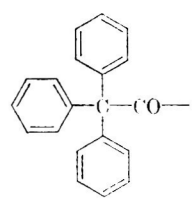
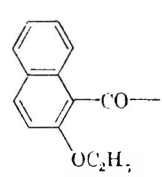
All the penicillins used caused accumulation of relatively large amounts of keto acids and of ultraviolet-absorbing materials in the culture fluids (Table 2). The latter effect in particular is indicative of leakage, probably resulting from damage to the cell membrane.

Annulment by pantoyl lactone of the growth- and division-inhibitory effects of penicillins

Pantoyl lactone prevents filament formation by benzylpenicillin when in the medium from the time of inoculation, and also annuls growth-inhibitory effects when present at the proper concentration relative to the penicillin concentration (Grula & Grula, 1962); higher concentrations are toxic. Pantoyl lactone prevents benzylpenicillin-induced release of ultraviolet-absorbing materials into the growth medium, and greatly decreases the accumulation of keto acids (Grula & Grula, 1964). In the absence of any inhibitory agent pantoyl lactone has little effect on growth or may decrease it slightly. Two lines of evidence indicate that the effects of pantoyl lactone do not result from a direct inactivation of penicillin. These are: (1) pantoyl lactone annuls the effects of a variety of chemically unrelated division-inhibitory compounds; (2) pantoyl lactone can cause division of long bacterial forms which have been grown in the presence of penicillin for 12–16 hr.

The effects of all the penicillin derivatives tested in the present work were annulled by pantoyl lactone in a very similar manner (Tables 2, 3); at slightly toxic or near non-toxic concentrations of a penicillin, pantoyl lactone inhibited growth.

Table 1. *Effect of penicillin derivatives on growth and cell length of Erwinia*

Compound	Structure of side chain	Concentration required to produce 50% inhibition of growth (mμmole/ml.)	Average bacterial length estimated at 50% inhibition of growth (μ)	Bacterial length standard deviation
Benzylpenicillin		90	40	10
DL-α-Aminobenzylpenicillin (D:L = 6:4)		10	45	9
D-α-Aminobenzylpenicillin		12	36	8
DL-3-Chloro-α-aminobenzylpenicillin		13	34	6
Methicillin		12	85	20
6-Aminopenicillanic acid		80	45	10
Cloxacillin		88	70	9
Triphenylmethylpenicillin		270	55	12
2-Ethoxy-1-naphthylpenicillin		310	86	10

In some cases it was possible to distinguish different degrees of activity by pantoyl lactone with regard to division inhibition. For example, with D-ampicillin and 6-aminopenicillanic acid, bacteria grown with the penicillin alone in each case were about the same length. Added pantoyl lactone annulled growth-inhibition with each penicillin (when used at a toxic concentration) to a similar degree, yet the bacteria were much shorter with 6-aminopenicillanic acid. In general, the annulment by pantoyl lactone of division-inhibition by the aminobenzylpenicillins was poor.

Table 2. *Prevention by pantoyl lactone of penicillin-induced accumulation of keto acids and ultraviolet-absorbing materials*

Penicillin (mμmole/ml.)	Keto acids (μg./mg. dry wt. bacteria)		Protein* (mg./mg. dry wt. bacteria)		Nucleic acid* (mg./mg. dry wt. bacteria)	
	Control	Pantoyl lactone (0·042 M)	Control	Pantoyl lactone (0·042 M)	Control	Pantoyl lactone (0·042 M)
Benzylpenicillin 90	500	36	0·44	0·085	0·31	0·029
DL-α-Aminobenzylpenicillin 35	290	45	0·80	0·18	0·43	0·05
D-α-Aminobenzylpenicillin 76	230	90	1·2	0·60	0·51	0·24
3-Chloro-α-aminobenzylpenicillin 33	200	29	0·44	0·12	0·13	0·05
Methicillin 76	1800	50	1·3	0·52	1·4	0·10
Cloxacillin 35	910	38	0·43	0·11	0·22	0·034
6-Aminopenicillanic acid 65	230	50	0·34	0·09	0·23	0·036
Triphenylmethylpenicillin 60	1240	12	0·44	0·08	0·08	0·05
2-Ethoxy-1-naphthylpenicillin 326	280	56	0·24	0·07	0·11	0·033

* Determined from 260/280 mμ extinction ratio of culture supernatant fluids.

Table 3. *Pantoyl lactone annulment of growth-inhibition and division-inhibition of an Erwinia caused by penicillin derivatives*

Penicillin (mμmole/ml.)	Growth*		Average bacterial length (μ)		Ratio of average length control/pantoyl lactone (0·042 M)
	Control	Pantoyl lactone (0·042 M)	Control	Pantoyl lactone (0·042 M)	
Benzylpenicillin 90	0·25	0·68	55	7	7·8
DL-α-Aminobenzylpenicillin 35	0·20	0·64	49	11	4·4
D-α-Aminobenzylpenicillin 76	0·15	0·21	30	22	1·4
3-Chloro-α-Aminobenzylpenicillin 33	0·24	0·77	37	7	5·3
6-Aminopenicillanic acid 65	0·80	0·68	37	3·5	10·6
Cloxacillin 35	0·96	0·80	38	3·5	10·8
Methicillin 76	0·07	0·60	100	24	4·1
Triphenylmethylpenicillin 60	1·10	0·95	13	2·7	4·9
600	0·09	0·54	110	36	3·1
2-Ethoxy-1-naphthylpenicillin 326	0·48	0·69	110	13	8·5

* Extinction (E_{540}) at 16 hr.

DISCUSSION

The similarity in the biological effects of the penicillins tested on this Erwinia supports the theory that the basis of penicillin action lies in the nucleus of the molecule, in agreement with the conclusions of Cooper (1956) and others. Since the side chain is not essential for the basic activity of penicillin, its influence on activity as observed by Boman & Eriksson (1963) may be a reflexion of its influence on the rate of binding at the active site and on the strength of the bond formed. After binding, the activities of different penicillins are similar. Rolinson (1962) reached the same conclusion after comparing the bactericidal activity of different penicillins in non-limiting concentrations. Apparent differences in activity he believed to result from differences in affinity of the molecule for the active site. To our knowledge little has been done on the influence of the side chain on the rate of uptake, or on the firmness of attachment at the active site. Molnberg & Johnson (1958) found that cephalosporin N was bound much less rapidly than benzylpenicillin. While we recognize that differences in permeability of an organism to different penicillins may be a factor in determining their relative activities, other factors must transcend permeability as the source of these differences. If permeability alone were the basis of our results, the effects on growth and bacterial length should parallel each other, which they do not (Table 1).

It is reasonable that the influence of the side chain should depend in part on the chemical nature of the receptors at the active site (see Cooper, 1956) and on permeability characteristics of the cell membrane and that different bacteria will show differences in the relative activities of different penicillin derivatives. This is illustrated by a comparison of our results with those of Boman & Eriksson (1963). With two compounds, 6-aminopenicillanic acid and methicillin, we observed a far higher activity with the Erwinia than Boman & Eriksson did with *Escherichia coli*. The potency of methicillin with the Erwinia indicates that a bulky side chain on the molecule does not necessarily preclude a high degree of activity; cloxacillin (not used by Boman & Eriksson), another penicillin with a large side chain, showed high activity for the Erwinia. On the other hand, two penicillins with bulky side chains, triphenylmethylpenicillin and 2-ethoxy-1-naphthylpenicillin, were only about one-twentieth as toxic as methicillin.

The possibility that side-chain structure may affect the intrinsic activity of the molecule must be recognized. The greater toxicity of the aminobenzylpenicillins than benzylpenicillin, originally observed with some Gram-negative bacteria (Rolinson & Stevens, 1961), may be an example; this higher activity also applies to specific enzyme systems (Rogers & Mandelstam, 1962). In our case, the relatively poor annulment by pantoyl lactone of the inhibitions by the aminobenzylpenicillins could be interpreted as indicating increased intrinsic activity toward the Erwinia resulting from the presence of a primary α-amino group in the side chain.

Collins & Richmond (1962) proposed that the structural similarity between *N*-acetylmuramic acid and penicillin might serve as a basis for antibiotic action. With molecular models, they showed that three atoms in the penicillin molecule, viz. O of the amide linkage of the side chain, N of the β-lactam ring, and O of the carboxyl group, all capable of forming hydrogen bonds, correspond in relative position to three atoms of *N*-acetylmuramic acid, also capable of forming hydrogen

bonds. The action of penicillin might then be explained on the basis that the antibiotic occupies the site normally occupied by *N*-acetylmuramic acid on an enzyme. Alteration of the relative positions of these atoms in penicillin is sufficient to destroy activity; an example is the β-lactam ring opening caused by penicillin β-lactamase (Pollock, 1962). However, removal of oxygen from the amide link of the side chain does not destroy activity, or even necessarily decrease it 1000-fold, as stated by Collins & Richmond (1962). In the case of the Erwinia, 6-aminopenicillanic acid (no side chain) was as toxic and as effective to inhibit division as benzylpenicillin. Our results with the Erwinia do not support the hypothesis of Collins & Richmond (1962), nor do the results of Hugo & Russell (1960), who observed that the concentration of 6-aminopenicillanic acid needed to induce spheroplasts in Gram-negative bacteria was only about twice as great as that for benzylpenicillin.

That inhibition of mucopeptide synthesis is the sole action of penicillin is a hypothesis not adequate to explain all the effects of penicillin; the partial independence of growth-inhibition and division-inhibition shown by the penicillins we have tested is a case in point. Grula & Grula (1964) showed that restoration of the ability to divide to penicillin-inhibited bacteria by adding pantoyl lactone was not accompanied by a restoration of cell-wall mucopeptide synthesis; division-inhibition is not a consequence only of inhibition of mucopeptide synthesis. The decrease in release of ultraviolet-absorbing materials, and in the accumulation of keto acids caused by adding pantoyl lactone may be because of protection afforded the cell membrane by this compound. The relationship of these effects of pantoyl lactone to its ability to prevent division inhibition is not known; however, evidence has been presented (Grula & Grula, 1964) that membrane damage occurs under most conditions of division-inhibition.

The ability of penicillin or its derivatives to inhibit bacterial division, as well as other biological activities, is a property of the β-lactam ring of the nucleus of the molecule. The effect of the side chain is not on the intrinsic activity, but on the affinity of the molecule for the active site. Factors independent of the penicillin which may affect binding, some of which may vary with the species (chemical nature of receptor sites, permeability) would be expected to influence the observed relative activities of various penicillins.

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Paper Chromatography as an Aid to the Identification of Nocardia Species

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SUMMARY

A simple method of detecting arabinose by paper chromatography in the cell-walls of aerobic actinomycetes is described. Mycobacteria and Nocardia species are rich in arabinose while saprophytic species of Streptomyces are deficient. Pathogenic Streptomyces species tend to fall between the two extremes but *Streptomyces somaliensis* is apparently nearly devoid of arabinose.

INTRODUCTION

A vast number of aerobic actinomycetes have been assigned to the genera *Nocardia* and *Streptomyces*. The majority of the latter are saprophytic organisms but three members, viz. *Streptomyces somaliensis*, *S. pelletieri* and *S. madurae* are frequent causes of mycetoma in some parts of the world. Whether they genuinely belong to the genus *Streptomyces* rather than to the genus *Nocardia* has been disputed. In human pathology two species, *Nocardia asteroides* and *N. brasiliensis*, have been completely accepted as important causes of mycetoma and other diseases. Unfortunately they can on occasion be difficult to distinguish from other actinomycetes. One criterion is acid-fastness, some measure of which Nocardia species frequently but not invariably possess. When suspected isolates are not acid-fast, identification can be very difficult and if it can be shown that the detection of arabinose serves as an acceptable substitute the problem would be considerably eased.

Cummins & Harris (1956) demonstrated that the cell-walls of Nocardia and Mycobacterium in contrast with those of Actinomyces and Streptomyces contained large amounts of arabinose. They later extended this study (Cummins & Harris, 1958) to *Actinomyces israelii* (11 strains), *A. bovis* (10 strains), Nocardia (5 strains), Mycobacteria (5 strains) and a few fungi. The cell-walls of strains of both Nocardia and Mycobacterium were found to be rich in arabinose, whereas none of the other organisms, with the exception of a strain of *A. bovis* (NCTC 4501) contained any. Bishop & Blank (1958) have demonstrated that D-arabinose is present in the cell-walls of *Nocardia asteroides*. Becker, Lechevalier, Gordon & Lechevalier (1964) have drawn attention to the detection of LL- and meso-diaminopimelic acid in cell-wall hydrolysates as a means of distinguishing between Nocardia and Streptomyces species. They also used paper chromatography.

In general, some isolates of both Nocardia and Streptomyces can be extremely difficult to place correctly and acid-fastness in particular is a fallible criterion. The following method of arabinose detection by paper chromatography is put forward as a method simple enough to fit into routine laboratory methods.

METHODS

Organisms were grown in nutrient broth containing 1% dextrose (25 ml. lots of medium in 50 ml. flasks) at 30° in shake cultures for a week, except in the cases of *Thermopolyspora polyspora* and *Micromonospora vulgaris* where the temperature was raised to 45°. Equally good results can be had from still cultures (50 ml. medium/100 ml. flask), but these call for incubation extended to 2 or 3 weeks. The growth was centrifuged out and washed once in water; the deposit was suspended in 3 ml. of 5% sulphuric acid and autoclaved at 5 lb/sq. in. for 20 min. Since Cummins & Harris (1958) advised disintegrating the cells in a Mickle shaker we at first made use of sonic disintegration before acid hydrolysis but it was found that this step could be omitted without impairment of the result. After autoclaving, 1 g. barium hydroxide was dissolved in the hydrolysate followed by 2 drops of universal indicator. Excess alkali was neutralized by the cautious addition of 5% sulphuric acid, or simply by bubbling carbon dioxide through the mixture. The barium precipitate and the remnants of the organisms were removed by centrifugation and the supernatant fluid reduced by drying over phosphorus pentoxide, or by evaporation under reduced pressure, from about 2·5 ml. to 0·3–0·5 ml.

Two methods of chromatography, both described in *Chromatographic Methods* (Stock & Rice, 1963) were used:

(1) *Circular chromatography*

17 cm. circular sheets of Whatman no. 2 filter paper were used. From the concentrated extracts, spots were deposited on the circumference of a circle of 2·5 cm. diam. at the centre of the filter paper; each sheet of paper could conveniently accommodate six spots of which one was normally a marker of 1% arabinose. Ribose can be mixed with the arabinose and/or with the hydrolysates if any confusion with the former sugar is anticipated. A hole was punched in the centre of the paper with a pointed glass rod and a wick of tightly rolled filter paper inserted. The chamber consisted of the lids of two 15 cm. glass Petri dishes with a layer of solvent (butanol + acetic acid + water; 4 + 1 + 5) in the bottom lid. No attempt was made to conserve solvent or prevent evaporation; the whole apparatus can be set up in a few minutes and the layer of solvent presumably saturates the atmosphere in the chamber very quickly. The paper was sandwiched between the two Petri dish lids, placed rim to rim, with the wick, which must be tightly rolled and a good fit in the hole, resting in the solvent. When the solvent had run for 2 hr, the paper was cautiously dried high over a Bunsen burner after removal of the wick. The drying could equally well be carried out in a suitable oven.

(2) *Descending chromatography*

Chromatography was carried out in a tank on strips of Whatman 3 MM paper 5 cm. wide using the same hydrolysates and solvent as in the circular method. The solvent was allowed to run for about 18 hr and the paper dried as above.

In both cases the dried paper was sprayed with aniline hydrogen oxalate (0·9 ml. re-distilled aniline in 100 ml. 0·1 M-aqueous oxalic acid: Horrocks & Manning, 1949). This reagent gives striking results with pentoses, notably a characteristic bright red for arabinose. Ribose gives an identical colour with this reagent, but

Cummins (1962) states that ribose very rarely occurs in these hydrolysates and then only in very small quantities. Nevertheless, trial spots of both arabinose and ribose, together with hydrolysates of organisms, were run in the manner described. Ribose moved consistently further than arabinose and could clearly be distinguished from it by its R_F (Pl. 1, figs. 1, 2). In no case was ribose detected in a hydrolysate of the organism tested.

After spraying, the paper was again dried in the same way. Arabinose appeared as a bright red crescent or spot and there was usually also a brown hexose band nearer the origin. The R_F of the spots can easily be measured as the red component of the universal indicator added after hydrolysis moves with the solvent front and when dry, marks it well.

RESULTS

The method was applied to *Nocardia asteroides* (3 strains), *N. brasiliensis* (3 strains), 6 other Nocardia strains, *Streptomyces madurae* (2 strains), *S. pelletieri* (2 strains), *S. somaliensis* (4 strains), 8 saprophytic Streptomyces strains, *Thermopolyspora polyspora* (1 strain), *Micromonospora vulgaris* (1 strain) and 5 saprophytic Mycobacterium strains.

As far as arabinose content is concerned these organisms fall into three clear-cut groups:

(1) Those containing a great deal of arabinose—all species of Nocardia and Mycobacterium tested.

(2) Those containing less, but still an appreciable amount of, arabinose, *Streptomyces madurae*, *S. pelletieri* and *T. polyspora*.

(3) Those containing no, or only a very faint trace of, arabinose, *Streptomyces somaliensis*, *Micromonospora vulgaris* and the saprophytic Streptomyces.

These results in so far as they are comparable are similar to those achieved by Cummins & Harris (1956, 1958) but do not entirely tally with those published by Kwapinski (1964) who detected arabinose in, *inter alia*, Actinomyces but failed to do so in *Streptomyces madurae* and *S. pelletieri*. The culture which he describes as *Nocardia madurae* NCTC 1070 is according to the records of the Mycological Reference Laboratory a culture of *S. somaliensis* in which absence of arabinose is agreed.

The results are also in agreement with those of Becker *et al.* (1964) using diaminopimelic acid as a marker. They placed *Streptomyces somaliensis* with the other Streptomyces species in general because of the presence of LL-diaminopimelic acid but they placed *S. pelletieri* and *S. madurae* with the Nocardia species because of the presence of meso-diaminopimelic acid.

DISCUSSION

Most species of the genus *Streptomyces* are clearly differentiated from species of *Nocardia* and *Mycobacterium* by the arabinose content of the cell-walls, the apparent anomalies being the three pathogens, *S. madurae*, *S. pelletieri* and *S. somaliensis*, whose true taxonomic position has been disputed. These three organisms were generally, but not constantly, regarded as members of the genus *Nocardia* till Gonzalez-Ochoa & Sandoval (1955) transferred them to *Streptomyces* on the basis of their spore formation under certain conditions of culture. Mackinnon & Arta-

gaveytia-Allende (1956) considered this question unsettled and it has since been shown that *Nocardia asteroides* (Gordon & Mihm, 1958) can produce spores indistinguishable from those associated with the genus *Streptomyces*. It is also interesting to note that Gonzalez-Ochoa & Vazquez-Hoyos (1953) described a serological separation that agrees fairly closely with our separation on the basis of the arabinose content; their four groups were:

(1) 'Bovis' group, anaerobic Actinomyces; species, *N. asteroides* and *N. brasiliensis*.

(2) 'Somaliensis' group; only *S. somaliensis*.

(3) 'Madurae' group; *S. madurae* and *S. pelletieri*.

(4) 'Paraguayensis' group, saprophytic Streptomyces species.

Descending chromatography gives better separation of spots than circular chromatography but takes longer and calls for somewhat more sophisticated apparatus. The very simple circular method described here serves to make an adequate distinction between the presence and absence of arabinose in cell-wall hydrolysates of the organisms under consideration. Possible confusion due to the presence of ribose can be avoided by running the hydrolysates with and without a ribose marker. The ribose appears as a spot beyond the adjacent arabinose spots.

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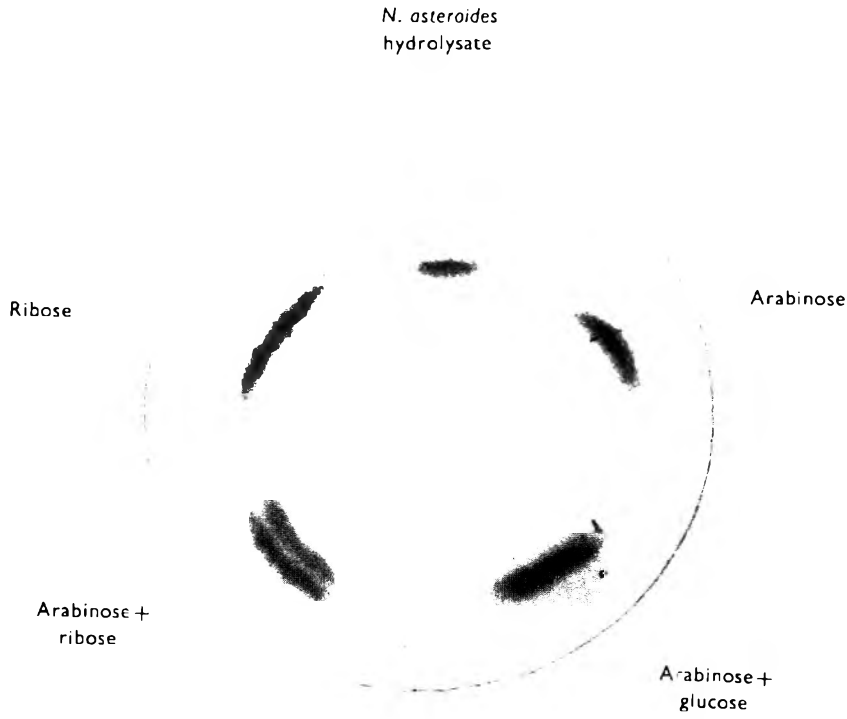
N. asteroides
hydrolysate
Ribose
Arabinose
Arabinose +
ribose
Arabinose +
glucose

Fig. 1

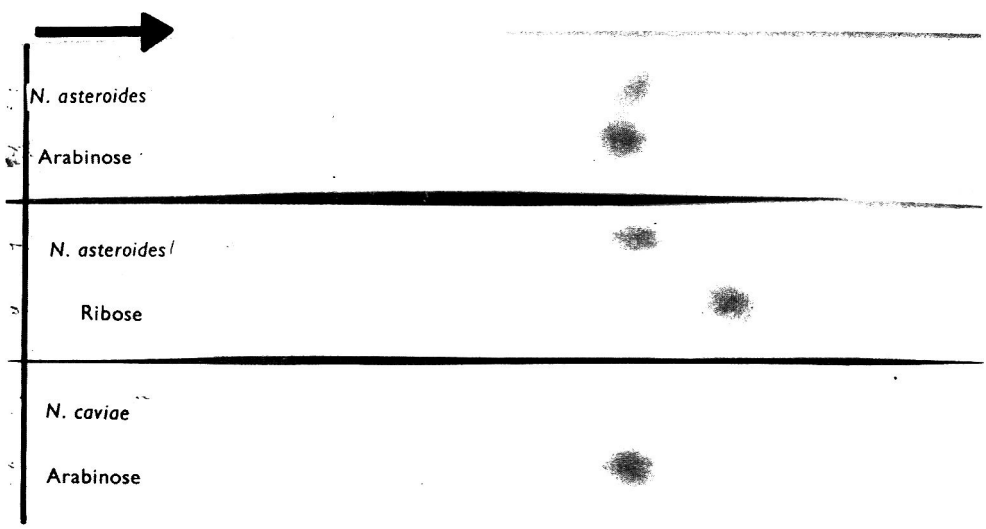
N. asteroides
Arabinose
N. asteroides
Ribose
N. caviae
Arabinose

Fig. 2

EXPLANATION OF PLATE

Fig. 1. The dark crescents mark the movement of several sugars and of a hydrolysate of *N. asteroides*.

Fig. 2. The dark spots mark the movements of two pentoses and two species of *Nocardia* (half natural size).

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The Urease Activity of Fluorescent Pseudomonads

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SUMMARY

Media frequently used for the detection of urease activity were found to be unsuitable for fluorescent pseudomonads since the presence of free ammonia was found to suppress the formation of urease in growing cultures. Incubation of cultures for 40 hr at 25° in a low concentration Casitone + yeast-extract + glucose medium, followed by the addition of urea to 0·2 % (w/v), resulted in the development of an alkaline shift in the medium after further incubation for a few more hours at 37°. This method gave good results in the detection of urease activity.

INTRODUCTION

De Turk (1955) recorded the adaptive (inducible) formation of urease in washed suspensions of *Pseudomonas aeruginosa* harvested from nutrient broth. Nevertheless Klinge (1959, 1960) defined the genus *Pseudomonas* as 'urease negative', Brisou (1958) found no ureolytic strains among pseudomonads from various sources and Rhodes (1959) was unable to show with certainty the possession of urease by any of 169 fluorescent pseudomonads which she examined. However, Lysenko (1961), who used essentially the same medium as Rhodes (1959), described pseudomonad isolates which apparently possessed urease activity. These workers and others used media either originally devised for use with the Enterobacteriaceae or inorganic media containing urea with or without another organic carbon source. The present work was done to evaluate the various urea media described in the literature for urease detection, and to determine whether fluorescent pseudomonads do possess urease activity and, if so, under what cultural conditions the enzyme might best be demonstrated.

METHODS

Bacteria. Fifty isolates classified as belonging to the genus *Pseudomonas* from hen eggs, soil, river and ditch water, milk, and chicken droppings, were examined. All were Gram-negative, polarly flagellate rods which grew well at 25° but not at all at 42°. All oxidized glucose, were oxidase positive, penicillin resistant and produced pyoverdine (fluorescin) but not pyocyanine on the appropriate media proposed by King, Ward & Raney (1954). Nine distinct bio-groups had been recognized among 30 of the isolates on the basis of more than 70 biochemical features (Stewart, 1964); by using the criteria proposed by Klinge (1959, 1960), 24 of these 30 isolates were assignable to *P. putida* and the other 6 to *P. fluorescens*. Similarily 15 of the other 20 isolates were classified as *P. putida* and the remaining 5 as *P. fluorescens*. The organisms were maintained on nutrient agar slopes.

Basal modified urease (MU) medium. Casitone (Difco), 0·02 g.; yeast extract (Difco), 0·02 g.; glucose, 0·05 g.; NaCl, 0·3 g.; K_2HPO_4, 0·02 g.; distilled water 98 ml.; mixed indicator solution, 2 ml. Adjusted to pH 7·4. Sterilized for 15 min. at 120°.

Glucose was chosen in preference to galactose, which was used by Rhodes (1959), because manometric studies showed that glucose was more rapidly oxidized by the isolates. The buffer salt (K_2HPO_4) was kept at a low concentration to allow a more rapid increase in pH value with base production. The mixed indicator system proposed by Singer (1950) was chosen since its green colour at pH 7·4 changes to blue at about pH 8·2 and to a distinct violet at about pH 9·0, thus allowing relative degrees of base production to be assessed. It consists of three indicator solutions prepared by dissolving 0·20 g. of bromothymol blue, cresol red and thymol blue in 6·4 ml., 10·6 ml. and 8·6 ml., respectively, of NaOH (0·05 N) and adding 100 ml. of distilled water to each solution; the three solutions are then mixed in the proportions 12·5:4:10 by vol.

Chemicals. Analar grade NH_4Cl, $(NH_4)_2SO_4$ and urea (Hopkin and Williams) and 40% (w/v) sterile urea solution (Oxoid) were used.

Delivery of 0·02 ml. amounts. Platinum-tipped dropping pipettes (Astell) were used.

Assay of urease activity in washed suspensions. To 0·40 ml. of a 0·25% (w/v) solution of urea (Hopkin and Williams) in veronal buffer (0·04 M, pH 7·2) was added 0·10 ml. of bacterial suspension containing the equivalent of 200 μg. dry wt. bacteria. The ammonia released after incubation for 1 hr at 30° was estimated by the Berthelot reaction on 0·10 ml. of reactant mixture with UN Kit reagents (Hyland Laboratories, California). Endogenous and substrate ammonia were similarily determined on appropriate incubated mixtures. NH_3-N values were calculated from a standard curve prepared from $(NH_4)_2SO_4$ solutions in ammonia-free distilled water.

Urease activity (qNH_3-N) was expressed as μg. NH_3-N released under the assay conditions by equiv. 1 mg. dry wt. bacteria/hr at 30°.

Dry weights of pseudomonad suspensions. These were determined by nephelometry and the use of a calibration curve.

RESULTS

Behaviour of the pseudomonad isolates in various urea-containing media

The following media were prepared: A (Christensen, 1946); B (Brisou, 1958); C (Stuart, van Stratum & Rustigian, 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959); MU medium (present paper). Their compositions are given in Table 1. The various media were inoculated, with a straight wire, from nutrient agar cultures of the 50 isolates and incubated at 25°. Daily for the first 14 days and then every few days for a total of 42 days, the tubes were examined for growth and change in pH value. The results (Table 2) clearly showed that media B, C and D were unsuitable for the growth of all but a few of the isolates. Most of the isolates grew in medium E although only 3 of 45 of these gave an alkaline reaction. All the isolates grew well in medium A to produce in most cases (45/50) a slow alkaline reaction. Forty of these 45 isolates, however, gave a similar reaction in this medium even when the urea was omitted, a phenomenon earlier reported by Rhodes (1959), and one which renders medium A useless in testing such organisms for urease activity.

Table 1. *Urea-containing media*

Composition of the five urea-containing media examined: A (Christensen, 1946); B (Brisou, 1958); C (Stuart *et al.* 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959); MU (present paper)

Constituent	Percentage (w/v) composition in medium: A	B	C	D	E	MU
Peptone (Oxoid L37)	0·1	—	—	—	—	0·02 (Casitone)
$NH_4H_2PO_4$	—	—	—	—	0·1	—
Yeast extract (Difco)	—	—	0·01	—	—	0·02
Glucose	0·1	—	—	—	—	0·05
Galactose	—	—	—	—	0·1	—
Ethanol	—	—		1·0	—	—
KH_2PO_4	0·2	0·1	0·91	0·1	—	—
K_2HPO_4	—	0·1	—	0·1	—	0·02
Na_2HPO_4	—	—	0·95	—	—	—
NaCl	0·5	—	—	0·5	—	0·3
KCl	—	—	—	—	0·02	—
$MgSO_4.7H_2O$	—	—	—	—	0·02	—
Agar	2·0	—	—	—	—	—
Urea (Oxoid)	2·0	2·0	2·0	2·0	2·0	[0·2]*
Indicator	PR	BTB	PR	PR	PR	S
Initial pH	6·8	7·0	6·8	7·0	7·0	7·4

PR = Phenol red; BTB = Bromothymol blue; S = Singer (1950).
* Added after culture incubated at 25° for 44 hr.

Table 2. *Behaviour of the 50 pseudomonad isolates in the urea-containing media*

Showing the proportion of isolates able to grow and to produce an alkaline reaction in media: A (Christensen, 1946); B (Brisou, 1958); C (Stuart *et al.* 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959)

Medium	Proportion able to grow	Proportion producing an alkaline reaction	Time for an alkaline reaction to develop (weeks)
A	50/50	45/50	3–4
B	8/50	8/8	3–4
C	5/50	0/5	—
D	7/50	7/7	3–4
E	45/50	3/45	2–3

Behaviour of the pseudomonad isolates in MU medium containing various concentrations of urea

Urea (Oxoid) was added aseptically to batches of sterile basal MU medium (pH 7·4) to final concentrations (%, w/v) of: 0, 0·05, 0·1, 0·2, 0·5, 1·0, 2·0. These media were dispensed in 2 ml. amounts into test tubes (100 mm. × 10 mm.) and inoculated with 0·02 ml. of dilute suspensions of organisms in $\frac{1}{4}$-strength Ringer's solution prepared from nutrient agar cultures of 6 of the isolates (3 classified as *Pseudomonas putida*, 3 as *P. fluorescens*). These cultures were incubated for 14 days at 25° with daily examination for growth and change in pH value.

The 6 isolates grew well in all seven media; the results with one of them (S. 60) typical of the 6, are shown in Table 3. It appeared that urea concentrations greater

than 0·2 % in the MU medium delayed the change to a more alkaline pH value. All 50 isolates were then tested by growth in the 0·2 % urea MU medium; they all produced a blue colour within 7 days and a violet one within 12 days. In the MU medium without urea, all the isolates caused a slight but permanent acid change (yellow-green, pH 6·5) after incubation for a day or two.

Table 3. *Reactions of pseudomonad strain S. 60 in the MU media*

The time (in days) for the pH value to increase to 8·2 (indicator blue) and to 9·0 (indicator violet) in the MU media containing (%, w/v): 0, 0·05, 0·1, 0·2, 0·5, 1·0, 2·0 urea, inoculated with strain S.60 and incubated at 25°.

Percentage (w/v) urea in medium	Days for alkaline reaction to develop	
	pH 8·2 (blue)	pH 9·0 (violet)
0	n. al.*	n. al.
0·05	3	5
0·1	3	5
0·2	3	5
0·5	4	6
1·0	5	10
2·0	5	10

* n. al. = no alkaline reaction developed within 14 days.

Table 4. *Urease activities (qNH_3-N) of pseudomonad strain S. 1 incubated in MU media with and without urea*

Age of culture (days)	qNH_3-N of bacteria harvested from: MU medium	MU medium + 0·2 % urea	MU medium + 2 % urea
1	5·1	0·4	0·3
2	25·3	1·2	0·5
4	32·0	4·5	0·9
6	84·1	4·4	1·2

The development and activity of pseudomonad urease in MU media

Modified basal MU medium with 0·4 % (w/v) K_2HPO_4 and without indicator was prepared. Batches containing: no urea, 0·2 % (w/v) urea (Oxoid) and 2 % (w/v) urea (Oxoid), respectively, were dispensed in 100 ml. amounts in round bottles of 230 ml. capacity. A dilute suspension of one of the pseudomonad isolates (S. 1) was inoculated (1 ml.) to each of four bottles of the three MU media (0, 0·2, 2 % urea). After incubation for 1, 2, 4 and 6 days at 25°, the crop of isolate S. 1 was harvested by centrifugation from, on each occasion, one bottle of each of the three media. The various lots of deposited organisms were washed twice in distilled water, each resuspended to 1 ml. in veronal buffer (0·04 M, pH 7·2) and held at −20° for urease assay. The urease activities (qNH_3-N) of the suspensions are shown in Table 4. The bacteria grown in the absence of urea showed significant urease activity which increased greatly with the time of incubation of the cultures. Very little urease was formed in the bacteria grown in the 0·2 % urea medium and even less in the 2 %

urea medium. It was concluded that urea added to MU medium exerted a considerable inhibitory effect on the formation of urease.

The effect of urea and of ammonia on the development of urease

Organisms of pseudomonad strain S. 1 were harvested after growth for 6 days in MU media containing (w/v): (1) 0·0038 % NH_4Cl (equivalent to 10 μg. NH_3-N/ml.); (2) 2 % Oxoid urea; (3) 2 % Hopkin and Williams urea (urea added as a Seitz-filtered solution). Urease activities of the organisms and the supernatant NH_3-N concentrations of the culture fluids before and after incubation were measured. It can be seen from Table 5 that little or no ammonia disappeared from any of the media during incubation. The results indicated that the low concentration of glucose (0·05 %, w/v) in the medium was insufficient to allow the assimilation of any more nitrogen than was already provided in organic forms in the peptone and yeast extract and that the presence of very low concentrations of unassimilable NH_3-N was sufficient to suppress urease formation by the growing bacteria.

Table 5. *Ammonia-N assimilation by, and urease activity of pseudomonad strain S. 1 grown in MU media with or without the addition of NH_4Cl or urea*

Urease activity (qNH_3-N) of organism S. 1 harvested from, and the NH_3-N concentrations in the culture supernatant fluids of, MU medium with and without urea.

Medium	NH_3-N (μg./ml.) At inoculation	NH_3-N (μg./ml.) After 6 days at 25°	qNH_3-N
MU (no addition)	1·0	0·9	104
MU + 0·0038 % NH_4Cl	10·1	9·7	< 1
MU + 2 % Oxoid urea	38·0	36·5	< 1
MU + 2 % Hopkin and Williams urea	3·0	14·0	< 1

Technique adopted for demonstrating urease activity in fluorescent pseudomonads

On the basis of the above results, the following procedure was used to examine urease production by the 50 pseudomonad isolates. The isolates were inoculated by a straight wire from nutrient agar cultures into 2 ml. of basal MU medium (in 100 mm. × 10 mm. tubes). After incubation for 40 hr at 25°, good growth was apparent in all the tubes and the pH values had decreased to about 6·5. To each tube was then added aseptically 0·02 ml. of 20 % (w/v) urea solution, the tubes were shaken and re-incubated at 37°. Alkalinity developed within a few hours in all tubes; in no case did the incubation period for a pH value of 9·0 (violet) to be reached exceed 6 hr.

In addition to these 50 isolates, all of 25 isolates of *Pseudomonas aeruginosa* from clinical specimens and milk were found by this technique to hydrolyse urea actively (with these organisms the initial incubation for growth was at 37°).

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Modes of Resistance to Benzylpenicillin and Ampicillin in Twelve Klebsiella Strains

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SUMMARY

Tube sensitivity tests show that, of twelve penicillins, ampicillin, benzylpenicillin and 6-aminopenicillanic acid were the most active when tested against medium-sized inocula (10^6 organisms) of twelve strains of penicillinase-producing Klebsiella (11 *Klebsiella aerogenes* and 1 *K. ozaenae*), which had been isolated from clinical material and were resistant to both ampicillin and tetracycline. An inoculum size effect was consistently noted when ampicillin and benzylpenicillin were tested against different inocula; the effect was significantly greater with ampicillin. The following parameters were measured for the strains, using both benzylpenicillin and ampicillin: magnitude of the inoculum size effect; inherent sensitivity (sensitivity of a small inoculum); rate of penicillin destruction; ability of penicillins to pass bacterial permeability barriers. From considerations of the relationships between these values, it has been concluded that penicillinase is primarily responsible for the observed resistance of only two of the twelve strains; although penicillinase and the lack of ability of penicillins to obtain free access into the bacterial cells add to the over-all penicillin resistance of the other ten strains, the primary reason for their penicillin resistance is neither their possession of penicillinase nor their permeability barrier: such resistance presumably reflects an innate lack of sensitivity of the cell wall synthesizing complex to inhibition by penicillins.

INTRODUCTION

Ampicillin, a 'broad spectrum' semisynthetic penicillin, has been shown to be active (Rolinson & Stevens, 1961) against several species of Gram-negative bacteria as well as against Gram-positive organisms; however, it has not proved to be effective, either in clinical or in laboratory studies, against some species of Gram-negative bacteria, notably *Klebsiella aerogenes* and certain strains of Proteus and Pseudomonas (Trafford *et al.* 1962; Barber & Waterworth, 1964). Such organisms have become relatively more important as hospital pathogens (Anderson *et al.* 1964) since the introduction of the 'penicillinase-stable' penicillins, methicillin and cloxacillin, which has temporarily checked the penicillinase-producing *Staphylococcus aureus*. It is of considerable interest, from a fundamental as well as from a clinical viewpoint, to determine why *K. aerogenes* is insusceptible to ampicillin therapy. In some cases it has been shown that the resistance of certain strains of Gram-negative bacteria is due, solely or in part, to the possession by these organisms of penicillinase (Percival, Brumfitt & de Louvois, 1963; Sutherland, 1964); these experiments were performed using relatively few laboratory strains. Now that it has been shown that some penicillinase-producing organisms destroy ampicillin only very slowly (Ayliffe,

1963; Hamilton-Miller, 1963*b*; Percival *et al.* 1963; Sabath & Finland, 1963; Smith, 1963; Smith & Hamilton-Miller, 1963), the mere possession of this enzyme by a particular bacterial strain can no longer be invoked, as it has been in the past, to explain fully the resistance to ampicillin therapy of that strain.

The genus *Klebsiella* has so far proved intransigent to ampicillin therapy (Trafford *et al.* 1962; Anderson *et al.* 1964), and this paper is concerned with some facets of this resistance in twelve strains, with particular reference to their *in vitro* lack of sensitivity to ampicillin and benzylpenicillin. A brief report of part of the work described here has been made (Hamilton-Miller, Smith & Knox, 1964).

METHODS

Bacterial strains: all the organisms used in these studies have been isolated from clinical material obtained in this hospital. *Klebsiella aerogenes* strains 1, 43, 366, 370, 373, 402, 407, 414, 415 and 418 were isolated, during 1961 and early 1962, in a clinical trial of ampicillin (Trafford *et al.* 1962); *K. aerogenes* 83 and *K. ozaenae* 61 were both isolated in 1964 from chest infections. The strains were identified and classified by means of their biochemical reactions as interpreted by Cowan, Steel, Shaw & Duguid (1960). Penicillinase activity was detected by means of a membrane method, and acylase activity excluded by an extraction technique; in most cases the hydrolysis product of benzylpenicillin was identified as benzylpenicilloic acid by chromatography, all as previously described (Hamilton-Miller, 1963*a*). Cultures were maintained upon agar slopes and stored at 4°; they were subcultured every 6 months.

Tube sensitivity tests: serial doubling dilutions, in a final volume of 1 ml., of the various penicillins were made in infusion broth (Southern Group Laboratories, Hither Green, London, S.E. 13); tubes were inoculated with one drop (0·02 ml.) of a suitable dilution of a 6 hr (37°) culture of the requisite coliform organism (such cultures usually contained about 5×10^7 bacteria/ml. before dilution). Tubes were incubated overnight (16 hr) at 37° and then read: the lowest concentration of penicillin which completely prevented growth was taken as the minimum inhibitory concentration. Inoculum sizes were counted by making serial decimal dilutions of the inoculum stock suspension, dropping 0·02 ml. on to blood agar, and counting the colonies after overnight incubation at 37°. The results of the tube sensitivity tests were interpreted as follows: the minimum inhibitory concentrations using small inocula were taken as measures of the 'intrinsic' resistance of each strain, while the ratio of the minimum inhibitory concentration using a large inoculum to that using a small inoculum was taken as a measure of the resistance due to penicillinase (the validity of this assumption is discussed in a later section).

Penicillins. Na benzylpenicillin (Crystapen) was obtained from Glaxo Ltd, Greenford, Middlesex; phenoxymethylpenicillin (penicillin V), K phenoxyethylpenicillin (Broxil, phenethicillin), K phenoxypropylpenicillin (Brocillin, propicillin), Na 2,6-dimethoxyphenylpenicillin (Celbenin, methicillin), α-aminobenzylpenicillin (Penbritin, ampicillin), Na 5-methyl-3-phenyl-4-isoxazolylpenicillin (oxacillin), Na 5-methyl-3-*p*-chlorphenyl-4-isoxazolylpenicillin (BRL 1577), Na 5-methyl-3-*o*-chlorphenyl-4-isoxazolylpenicillin (Orbenin, cloxacillin), and 6-aminopenicillanic acid were gifts of Beecham Research Laboratories Limited, Brockham Park, Surrey; phenoxybenzylpenicillin (Penspek, phenbenicillin) was a gift from The Distillers

Company (Biochemicals) Ltd, Speke, Liverpool; Na_2 3-carboxy-2-quinoxalinyl penicillin (quinacillin) was a gift from Boots Pure Drug Company, Nottingham.

Properties of penicillinases: these were investigated as previously described (Hamilton-Miller, 1963*a*), using the hydroxylamine assay. Cultures were incubated statically in infusion broth at 37° for 16 hr, harvested by centrifugation, washed and resuspended at a suitable concentration in 25 mM sodium phosphate buffer, pH 7·4; whole-cell and supernatant fractions of broth cultures were assayed for penicillinase activity (see Results). Disruption of bacterial suspensions was brought about, when desired, by treatment for 5 min. in the M.S.E. 60 W. ultrasonic disintegrator, using a probe of end diameter 1·9 cm., the suspension being cooled in an ice-water bath; preparations were then centrifuged at 30,000*g* for 35 min. at 4°.

Concept and definition of 'permeability factor': as penicillinase activity was strictly intracellular in the strains studied here (see Results) it is valid to assume that any increase in enzymic activity which follows disruption of cells is due to removal of substrate accessibility barriers which, in intact cells, limit the rate of entry of substrate into the interior of the cell. The value of this increase can be taken as a quantitative measure of the ease with which various substrates are able to penetrate the cell membrane. In this paper, such values are expressed as 'permeability factors', which were determined in the following way: a suitable suspension of washed cells was prepared, a portion disrupted and both intact and disrupted preparations assayed against the required substrate. The permeability factor is defined as the ratio of the rate of hydrolysis by the disrupted sample to the rate of hydrolysis by the intact preparation.

RESULTS

Location of penicillinase. Overnight broth cultures of all the strains were centrifuged, and the resuspended pellets assayed in parallel with the supernatant fractions. In no case was any penicillinase activity found in the extracellular fluid. Cell debris obtained by centrifugation of ultrasonically disintegrated suspensions (see 'Methods') was also devoid of penicillinase activity. In whole untreated cultures, therefore, it appears that penicillinase is a wholly intracellular enzyme, probably confined to the 'soluble cytoplasm' of the bacteria (Hamilton-Miller, 1963*a*). Solubilization of penicillinase activity was caused only by methods that completely destroyed the architecture of the bacterial cell (e.g. ultrasonic treatment, extrusion, freezing and thawing—see Hamilton-Miller, 1964). Methods which, while partially or completely destroying the permeability barriers which render penicillinase cryptic, nevertheless leave the cells viable and microscopically intact [namely culture in subinhibitory concentrations of various penicillins (Smith, 1963; Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1963*a*), or treatment with mM EDTA (Hamilton-Miller, 1964)], did not result in release of penicillinase into the extracellular fluid.

Properties of penicillinases. The following properties and characteristics of the penicillinases from each of the twelve Klebsiella strains were studied: specificity patterns, using twelve substrates; pH-activity characteristics; permeability of bacteria to various penicillins; apparent energies of activation; susceptibility to inhibition by substrate analogues; inducibility. These properties were similar in all respects to those described in previous studies involving only some of the strains (Hamilton-Miller, 1963*a*, 1964; Smith & Hamilton-Miller, 1963; Hamilton-Miller &

Smith, 1964). Table 1 shows absolute activities and permeability factors for the twelve strains using benzylpenicillin and ampicillin as substrates. The properties which are of particular relevance to the present study are as follows: ampicillin was hydrolysed more rapidly than was benzylpenicillin by both cell-free and whole-cell preparations from all the strains; enzymic activity was maximal between pH values 6 and 8; the affinity of the penicillinases for benzylpenicillin was uniformly high ($K_m < 50 \mu g./ml.$); the enzyme was in no case inducible.

Table 1. *Absolute activity and permeability factors for twelve Klebsiella strains against benzylpenicillin and ampicillin*

Activities expressed as mU (mμmoles substrate hydrolysed per min.,) using suspensions containing 10^9 bacteria/ml. (disrupted), in 25 mM sodium phosphate buffer pH 7·4, at 37°. Permeability factors (P) determined as described in 'Methods'.

		Benzylpenicillin		Ampicillin	
		Absolute activity	P	Absolute activity	P
K. aerogenes	1	10	6	19	7
	43	10·5	11	21·8	12·5
	83	1·7	1·5	2·5	1·5
	366	3180	11	6300	14
	370	22	8	37	8
	373	1360	9	2660	5
	402	7·6	3	15·8	2
	407	5·4	3	8·7	2
	414	3·8	2	6	2
	415	10·3	4	28	3
	418	20·6	4	35	4
K. ozaenae	61	22·3	2·5	40	3

Antibacterial activity of different penicillins. As the minimum inhibitory concentration of a penicillin acting against a penicillinase-producing organism is, under certain circumstances, dependent upon the size of the inoculum that is used (Luria, 1946; Knox & Smith, 1961; Hamilton-Miller *et al.* 1964), standardized conditions were employed for this series of experiments. An initial inoculum of about 10^6 organisms/tube was used. Eleven strains of Klebsiella were tested in this way, and minimum inhibitory concentrations were determined for eleven penicillins and 6-aminopenicillanic acid; results are shown in Table 2. The mean values of the minimum inhibitory concentrations of each compound for all the strains were then calculated, in an attempt to obtain a coherent idea of the relative antibacterial activities of this series of drugs; these figures are also shown in Table 2. It can be seen that benzylpenicillin, ampicillin and 6-aminopenicillanic acid are the most active compounds against Klebsiella strains, using this inoculum size. However, with the possible exception of *Klebsiella ozaenae* 61, the minimum inhibitory concentrations observed here are all very much greater than the concentrations that can be attained in the body during therapy.

Inoculum size effects. Some strains of *Klebsiella aerogenes* were found to show an inoculum size effect when tested against ampicillin or benzylpenicillin (Hamilton-Miller *et al.* 1964). The magnitude of this effect was measured for each strain using ampicillin and benzylpenicillin, by determining the ratio of the minimum inhibitory

concentration with a large inoculum (about 10^7 organisms/tube) to that with a small inoculum (about 10 organisms/tube). Preliminary investigations showed that varying the size of the inoculum between 100 and 10 organisms per tube did not alter the minimum inhibitory concentration; hence, for all practical purposes, an inoculum size of 100 organisms is equivalent to one of 10. Both are physiologically 'small' inocula in the sense that the minimum inhibitory concentrations obtained by their use reflect the inherent sensitivity of a strain. Table 3 shows the results of these experiments: the magnitude of the inoculum size effects obtained using ampicillin was found to be significantly greater ($t = 2{\cdot}781$ with 18 degrees of freedom, $P < 0{\cdot}02$) than that obtained using benzylpenicillin. *K. aerogenes* strains 366 and 373 have been omitted from this series, as their intrinsic resistance to ampicillin (minimum inhibitory concentration against small inocula = 12·5 mg./ml.) was so great that the full extent of the inoculum size effect could not be measured; it was also difficult to obtain a sufficiently concentrated solution of ampicillin in broth at the correct pH value with which to initiate the serial dilutions. The intrinsic resistance of these two strains to benzylpenicillin was also very great (minimum inhibitory concentration against small inocula = 1·5 mg./ml.).

Table 2. *Minimum inhibitory concentrations (μg./ml.) of twelve drugs for eleven Klebsiella strains*

Inoculum size was 10^6 organisms/tube.

	K. aerogenes										*K. ozaenae*	Mean
	1	43	83	366	370	402	407	414	415	418	61	values
Benzylpenicillin	1,000	500	31	2,500	125	100	125	250	80	220	16	450
Phenoxymethylpenicillin	2,000	1,000	125	2,500	500	625	900	500	400	500	32	825
Phenethicillin	4,000	2,000	1,000	10,000	2,000	2,500	2,000	2,000	1,000	2,000	125	2,600
Propicillin	4,000	2,000	500	10,000	2,000	1,250	2,000	1,000	1,000	1,000	125	2,260
Ampicillin	1,500	500	31	2,500	250	75	250	1,000	90	200	16	580
Phenbenicillin	2,000	500	250	1,000	500	400	500	2,000	300	400	32	715
6-aminopenicillanic acid	500	1,000	62	2,500	250	200	250	250	70	70	64	475
Methicillin	10,000	4,000	4,000	1,000	2,000	2,500	4,000	2,000	2,000	4,000	64	3,240
Oxacillin	2,000	2,000	1,000	800	1,000	625	1,000	500	500	1,000	64	955
Cloxacillin	1,000	1,000	1,000	400	1,000	1,250	1,000	1,000	1,000	1,000	64	885
BRL 1577	4,000	1,000	1,000	1,000	2,000	1,250	1,000	1,000	700		64	1,300
Quinacillin	> 8,000	> 8,000	> 4,000	> 4,000	> 4,000		> 8,000	> 8,000		> 4,000	> 4,000	> 4,000

On *prima facie* grounds it might be expected that, if resistance to benzylpenicillin and ampicillin is due solely to penicillinase activity, the more penicillinase activity a strain possesses, the greater should the resistance of that strain be. To test this hypothesis, the correlation was calculated between penicillinase activity against extent of penicillinase-type resistance displayed (as exemplified by the magnitude of the inoculum size effect). As can be seen from Table 4, rows A and B, there is no significant correlation between the penicillinase activity of a strain and the degree of penicillinase-type resistance shown by it, either toward benzylpenicillin or ampicillin.

The minimum inhibitory concentrations determined using the small inoculum can be taken as a measure of the inherent resistance of each strain to the penicillin tested. Again, in all cases except *Klebsiella aerogenes* 83 and *K. ozaenae* 61 (which had been selected for study originally because they showed a zone of inhibition around a 15μg. ampicillin disk on solid media), the concentration of any drug tested here required to inhibit the growth of a small inoculum was greater than the level of that drug that can be attained *in vivo* during therapy; i.e. the ten strains were inherently resistant to penicillins. However, it was found that a significant degree of correla-

Table 3. *Minimum inhibitory concentrations (m.i.c.) of benzylpenicillin and ampicillin (μg./ml.) for large (columns 2 and 5) and small (columns 2 and 6) inocula of ten Klebsiella strains*

Ratio (columns 4 and 7) of m.i.c. for large inoculum to m.i.c. for small inoculum gives a measure of amount of resistance due to penicillinase; m.i.c. for small inoculum gives a measure of inherent susceptibility of each strain.

		Ampicillin			Benzylpenicillin		
		Inoculum			Inoculum		
Bacterial strain		10^7	10	Ratio	10^7	10	Ratio
K. aerogenes	1	2,000	31	64	1,000	31	32
	43	1,000	31	32	1,000	63	16
	83	250	8	32	125	8	16
	370	2,000	16	128	1,000	31	32
	402	1,000	31	32	400	50	8
	407	1,000	16	64	500	31	16
	414	1,000	16	64	500	31	16
	415	200	13	16	400	28	16
	418	1,000	25	40	128	31	4
K. ozaenae	61	250	4	64	250	4	64
Mean		—	—	53·6	—	—	22·0
S.D.		—	—	31·6	—	—	17·2

Table 4. *Results of tests of correlation between various pairs of parameters measured in these experiments*

Rates of breakdown, and permeability factors, for benzylpenicillin and ampicillin taken from Table 1. Penicillinase type resistance taken from Table 3—ratio of minimum inhibitory concentrations obtained using large and small inocula; inherent resistance taken from Table 3—value of minimum inhibitory concentration using small inocula.

Correlation tested between	*t* value	Degrees of freedom	Significance level
(A) Penicillinase-type resistance to benzylpenicillin *vs.* rate of breakdown of benzylpenicillin	1·329	10	$P = 0{\cdot}22$ (not significant)
(B) Penicillinase-type resistance to ampicillin *vs.* rate of breakdown of ampicillin	0·0129	8	—
(C) Inherent resistance to benzylpenicillin *vs.* permeability factor to benzylpenicillin	2·553	10	$P = 0{\cdot}03$ (significant)
(D) Inherent resistance to ampicillin *vs.* permeability factor to ampicillin	1·801	10	$P = 0{\cdot}11$
(E) Inherent resistance to ampicillin *vs.* inherent resistance to benzylpenicillin	8·555	10	$P < 0{\cdot}001$ (highly significant)

tion existed between the inherent resistance of these strains and the ease with which benzylpenicillin penetrated through the permeability barrier into the bacteria (Table 4, row C). Although in the case of ampicillin the correlation between these factors is not significant at the 5% level (Table 4, row D) it is worthy of note that the probability of the degree of correlation observed being due purely to chance is only

11%. A further fact was observed, namely that the correlation between inherent resistance to ampicillin and inherent resistance to benzylpenicillin was highly significant (Table 4, row E).

DISCUSSION

It is interesting that both benzylpenicillin and 6-aminopenicillanic acid show activity approximately as great as ampicillin against resistant Klebsiella strains; against sensitive Gram-negative strains, ampicillin has been reported to be some ten times more active than benzylpenicillin (Rolinson & Stevens, 1961). Further, there have been other reports (see Hamilton-Miller, 1963*a*) of 6-aminopenicillanic acid having greater antibacterial activity than either benzylpenicillin or ampicillin, against certain coliform organisms.

The assumption has been made (see 'Methods' section) that the inoculum size effect observed in these experiments is due to penicillinase. It seems probable that this is the correct explanation for the phenomenon, for the following reasons:

(1) All these organisms possess β-lactamase activity, which is effective at the penicillin concentrations and pH values used; ampicillin is more rapidly hydrolysed than benzylpenicillin by whole cell suspensions of these organisms, which correlates with the inoculum size effect being of significantly greater magnitude with ampicillin than with benzylpenicillin.

(2) The microbiological studies of Hamilton-Miller *et al.* (1964), which showed that the inoculum size effect in *Klebsiella aerogenes* can be drastically reduced by *in vivo* inhibition of penicillinase activity, rule out the possibility that non-specific penicillin destruction is responsible for the inoculum size effect, and directly implicate penicillinase in this role.

(3) Three of the strains used in these studies (402, 415 and 418) have proved to have a homogeneous population structure in regard to their sensitivity both to benzylpenicillin and to ampicillin. (I am grateful to Mrs B. Heywood for performing these experiments).

While penicillinase has the capability of protecting large inocula against penicillins, the permeability barrier will be of particular importance when the sensitivity of individual bacterial cells is being considered: this inherent sensitivity has been shown to be low in most of the strains studied here, and bears no significant relationship to the extent of the permeability barrier to ampicillin. Hence it is very difficult to come to any other conclusion but that the inherent resistance of these strains to ampicillin is due to some other mechanism, such as an intrinsic lack of sensitivity on the part of the mucopeptide synthesizing enzyme complex to inhibition by this penicillin. The less permeable are the cells of a bacterial strain to benzylpenicillin, the more inherently resistant is that strain to this penicillin; it therefore seems possible that at least part of the reason for the high inherent resistance of most of the Klebsiella strains investigated here is the inability of benzylpenicillin to penetrate into the bacteria in adequate concentrations to cause bacteriostasis. The possession of a penicillinase which is capable of actively hydrolysing penicillins will serve to superimpose an additional resistance upon the inherent resistance. Moreover, the presence of a permeability barrier will actually enhance the effectiveness of the penicillinase (as pointed out by Percival *et al.* 1963): the rate of entry of the drug may be limited so that any which enters can be destroyed before it can cause damage in a

cell which possesses, in conjunction with an accessibility barrier, a low level of penicillinase.

But as the inherent resistance of most of these strains is more than adequate to enable them to survive in those levels of ampicillin or benzylpenicillin that can be obtained during therapy, the role of the penicillinase, in conjunction with the permeability barrier, seems to be merely to increase the 'lack of sensitivity' of these strains.

The effect of the presence of penicillinase upon the susceptibility of bacterial strains to penicillins can be measured directly, as has been done in the experiments described in this paper, by observation of the magnitude of the inoculum size effect, or indirectly, by inhibition of the penicillinase activity *in vitro* in intact, viable cells, as was done by Hamilton-Miller *et al.* (1964) and by Sutherland & Batchelor (1964), using methicillin to inhibit the enzyme. The results obtained from both avenues of exploration show that even in the absence of penicillinase-mediated penicillin destruction ten of these Klebsiella strains were inherently resistant to both benzylpenicillin and ampicillin. However, in view of the fact that in small inocula *Klebsiella aerogenes* 83 and *K. ozaenae* 61 were sensitive to ampicillin and benzylpenicillin in concentrations which can be attained *in vivo* during therapy, while in large inocula they were resistant to both drugs, it seems probable that these two strains owe their observed resistance to the fact that they possess penicillinase in conjunction with a permeability barrier; thus a parallel is shown with the results obtained by Percival *et al.* (1963) for *Escherichia coli* and Sutherland (1964) for *Aerobacter aerogenes*.

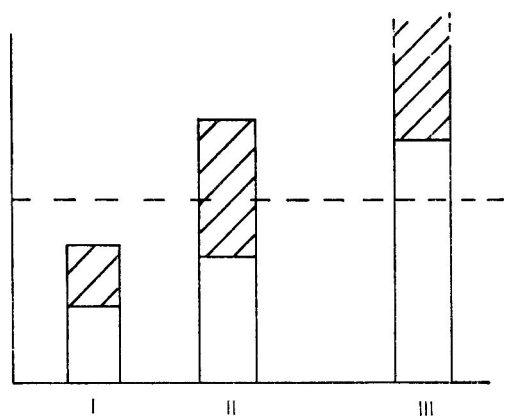

Fig. 1. The different classes of 'resistance' to a penicillin. Vertical axis represents minimum inhibitory concentration of drug; horizontal dotted line is the usually attained blood level of that drug. Height of bar represents degree of bacterial resistance: open part of bar is intrinsic resistance, hatched part is penicillinase type resistance. If height of bar exceeds blood level, strain is regarded as resistant. See text for characteristics of bacterial strains belonging to classes I, II and III.

Academically speaking, 'resistance' is impossible to define without picking some purely arbitrary, and therefore artificial, base-line (which is bound to be unsatisfactory). Clinically speaking, however, the base-line can be made the concentration of the drug which is usually attained in the serum of patients undergoing treatment; this definition will give satisfactory results. Consider the three situations which can

obtain in the case of penicillinase-producing organisms, in respect of their resistance to penicillins (Fig. 1).

Class I contains sensitive organisms, with low intrinsic resistance, and low or negligible amounts of penicillinase; such strains will show a zone of inhibition around a 'low level' penicillin disc.

Class II strains have a low or moderate intrinsic resistance, and a moderate or high level of penicillinase. This penicillinase activity tips the balance in such strains, rendering them resistant in large inocula; a zone of inhibition is usually seen around a 'high level' penicillin disc, but not around the 'low level' disc. Resistance in these organisms can be suppressed by inhibition of penicillinase activity (see Hamilton-Miller *et al.* 1964; Sutherland & Batchelor, 1964). *Klebsiella aerogenes* 83 and *K. ozaenae* 61 appear to fall into this category.

Class III consists of truly resistant organisms, of high intrinsic resistance; their penicillinase content is immaterial (though usually high). No zone of inhibition is seen around the 'high level' penicillin disc, and the penicillin is useless in the treatment of these cases, even if penicillinase activity can be suppressed. Ten strains in this series belong to this category.

The situation in regard to penicillin resistance in these bacteria offers a striking resemblance to the picture of resistance to chloramphenicol, where low levels of resistance in *Escherichia coli* were due to enzymic destruction of the drug, but higher levels were mediated by a genetic mechanism (Merkel & Steers, 1953); this genetic mechanism may very well be involved with the fact that the protein-synthesizing system, which is known to be the prime target for chloramphenicol, has been shown to be able to operate by an alternative route which is not susceptible to inhibition by chloramphenicol (Ramsey, 1958). Permeability factors have also been shown to be of paramount importance in the resistance of *E. coli* (Okamoto & Mizuno, 1964) and *Pseudomonas fluorescens* (Kushner, 1955) to chloramphenicol.

The possession of a penicillinase which increases the resistance of an already inherently resistant bacterial strain can have very little, if any, evolutionary significance *per se*: it may be purely coincidental that inherently resistant Klebsiella strains possess penicillinase activity. Indeed, it may be recalled that both Czekalowski (1950) and Abraham (1951) have suggested that penicillinase activity may be a coincidental manifestation of the mere possession of some functionally or structurally important protein molecule; it is possible that such a molecule may be involved as an integral part of the cell-wall-membrane complex in these bacteria (see Kaufmann, 1964). Further investigations have been carried out into the nature of the permeability barrier as it exists in some of these strains, and results will be reported in a subsequent paper.

I am grateful to Beecham Research Laboratories, Ltd., to Boots Pure Drug Co. Ltd., and to Distillers Co. (Biochemicals) Ltd. for generous gifts of penicillins. Professor R. Knox and Dr J. T. Smith gave much helpful advice and criticism, and Dr D. M. MacLaren kindly provided the organisms.

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γ-Glutamyl Transfer Reactions in Bacteria

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SUMMARY

γ-Glutamyl transfer activity was found to be widely distributed in different bacterial species. The γ-glutamyl transfer from glutathione to water and acceptors other than water was studied with cell-free preparations of *Proteus morganii*. In the absence of added acceptor, the γ-glutamyl residue was predominantly transferred to water; however, some transfer to the substrate, resulting in the formation of γ-glutamylglutathione, was detected. In the presence of acceptors (amino acids or peptides) all the γ-glutamyl residue was transferred to the added acceptor. The different reaction products were isolated and identified. Kinetics and properties of the γ-glutamyl transfer reaction were studied.

INTRODUCTION

γ-Glutamyl transfer from glutathione (GSH) to amino acids, peptides or water has been studied with enzymes of animal origin, mainly kidney (Hanes, Hird & Isherwood, 1950, 1952; Kinoshita & Ball, 1953; Fodor, Miller & Waelsch, 1953; Hird & Springell, 1954*a*, *b*; Ball, Revel & Cooper, 1956; Revel & Ball, 1959; Avi Dor, 1960; Binkley, 1961). Data on the γ-glutamyl transfer in bacteria are rather scarce. Only qualitative data on transfer of the γ-glutamyl moiety by washed suspensions and cell-free extracts of *Proteus vulgaris* have been reported (Samuels Talalay, 1954). The occurrence of γ-glutamyl transfer from glutathione in washed suspensions of different bacterial species was studied in order to gain information about the distribution and the activity of the enzyme. Since *P. morganii* showed pronounced transfer activity, cell-free extracts of this micro-organism were used extensively.

METHODS

Materials. Glutathione and all amino acids and peptides used were commercial preparations, most of them obtained from Nutritional Biochemicals Corp., Cleveland, U.S.A.

Preparation of washed bacterial suspensions. Bacteria were grown for 18 hr at 37° in Roux bottles containing nutrient agar. The bacteria were collected and washed 3 times with distilled water. Their optical density at 550 mμ was determined with a Coleman Junior Spectrophotometer. Optical density readings were converted to dry weight, by using a standard curve prepared for *Escherichia coli* B.

Determination of γ-glutamyl transfer with washed suspensions. The reaction mixture, 1·0 ml. total volume, contained: GSH, 10 μmole; phosphate buffer (pH 7·5), 60 μmole; and suspensions of washed bacteria equiv. to 2–4 mg. dry weight. Acceptor,

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when added, was 20 μmole. The reaction was run at 37° for 45 min. in a gas phase containing more than 99% (v/v) nitrogen. The acceptors used were glycylglycine and L-methionine.

Preparation of cell-free extracts. *Proteus morganii* was grown in nutrient broth with aeration for 18 hr at 37°. The organisms were collected in a Sharples Centrifuge, washed 3 times with distilled water and disintegrated for 10 min. in a 10 KC Raytheon Sonic Oscillator. The unbroken organisms and cell debris were removed at 40,000 rev./min. in a Spinco Model L Ultracentrifuge and the supernatant thus obtained served as the enzyme source. The enzyme was dialysed overnight, in the cold, against distilled water. Storage at −20° preserved the enzymic activity for several months. Protein was estimated by the biuret method according to Mehl (1945).

Determination of γ-glutamyl transfer with cell-free extracts. Unless otherwise stated, the reaction mixture, 1·0 ml. total volume, contained: GSH, 20 μmole; tris buffer (pH 8·5), 400 μmole; and 1·6 mg. protein. Acceptor, when added, 80 μmole of the L form or 160 μmole of the racemic compound. The reaction was run at 37° for 45 min. in a gas phase containing more than 99% (v/v) N_2.

Estimation of glutathione. The reaction was stopped and proteins were precipitated by diluting the reaction mixture with 20 vol. of 5% (w/v) metaphosphoric acid. The glutathione content of the filtrate was determined by the alloxan method of Patterson & Lazarow (1955). A glutathione standard curve and appropriate blanks were run concurrently.

Quantitative paper chromatography. The reaction mixture was treated with 2 vol. of hot ethanol containing 10 mM-*N*-ethylmaleimide. The protein precipitate was filtered off and 0·01 or 0·02 ml. samples were applied to Whatman 3 MM paper (for chromatography). The solvents used were propanol + water (80 + 20 by vol.), or tertiary butanol + formic acid + water (70 + 15 + 15 by vol.). Known amounts of the different markers were run simultaneously. The chromatograms (descending) were run for 16–18 hr at room temperature, developed with buffered ninhydrin (Haschen, 1956), sprayed with ethanolic $Cu(NO_3)_2$ reagent (Kawerau & Wieland, 1951) for colour stabilization and eluted with methanol (Hanson, Blech, Hermann & Kleine, 1959).

Methods for the isolation and identification of peptides. The reaction mixture, containing the peptide to be tested, was applied in a straight line 10 cm. from the bottom of the paper (Whatman 3 MM); usually 1 ml. reaction mixture was applied to each sheet (46 × 57 cm.). The chromatograms were run for 16–18 hr. The position of the peptides was determined by spraying small strips on both sides of the chromatogram. The areas containing the peptides were cut out and the compounds were concentrated and eluted according to Reith (1957). After two repeated isolation procedures, using propanol + water in the first and tertiary butanol + formic acid + water in the second, the peptide solution in 6 N-HCl was heated in a sealed ampoule for 10 hr at 105°. The hydrolysate was dried over $Ca_3(PO_4)_2$ and the products determined by paper chromatography. The actual values were obtained by converting the optical density readings (at 505 mμ) into μmole according to appropriate standards. Similarly treated filter paper was used as a blank.

Dinitrophenyl (DNP) derivatives of the isolated peptides were prepared according to Rosenthal & Tabor (1956). The purity of each derivative was tested by paper

chromatography. Hydrolysis and determination of the products was carried out as above.

Pyrrolidone carboxylic acid was tested for by the chlorine starch iodine method (Rydon & Smith, 1952) according to Ellfolk & Synge (1955).

RESULTS

Distribution of γ-glutamyl transfer activity in washed suspensions of various bacterial species

The search for γ-glutamyl transfer in the different bacteria used (Table 1) was done under conditions which have been found to be optimal for the transfer in washed suspensions of *Proteus vulgaris*. The acceptors used were L-methionine or glycylglycine and the disappearance of glutathione was measured by the alloxan method. Out of 16 different bacterial species tested 13 were found capable of hydrolysing glutathione, while the remaining three (*Escherichia coli*, *E. freundii* and *Staphylococcus aureus*) showed weak activity. The most active organisms were *Achromobacter delicatulum*, *P. morganii* and *P. vulgaris*. Addition of either of the acceptors increased the cleavage of the tripeptide by most of the bacterial species tested. *Proteus morganii*, because of its high activity, was chosen for a more extensive examination.

Table 1. *Distribution of γ-glutamyl transfer activity in washed suspensions of various bacterial species*

The different reaction mixtures, total volume 1·0 ml., contained: GSH, 10 μmole, or GSH, 10 μmole, and acceptor, 20 μmole; phosphate buffer (pH 7·5), 66 μmole; and washed bacterial suspensions 2–4 mg. dry weight. Incubation: 45 min. at 37°, under nitrogen. GSH cleavage was determined by the alloxan method.

Organism	Glutathione cleaved (μmole/mg. dry weight)		
	Without acceptor	Acceptor glycylglycine	Acceptor L-methionine
Aerobacter aerogenes	0·25	0·24	0·32
A. aerogenes 8303	0·13	0·11	0·17
Achromobacter delicatulum	3·20	3·70	3·20
Alcaligenes bookeri	0·11	0·40	0·40
A. faecalis	0·10	0·60	0·45
Bacillus mycoides	0·32	0·25	0·25
B. subtilis	0·72	0·76	0·92
Escherichia coli B	0·02	0	0·07
E. freundii	0·07	0·05	0·02
Klebsiella ozaenae	0·38	0·37	0·47
Proteus vulgaris (HX19)	1·10	1·42	1·42
P. morganii	1·78	2·48	2·48
Pseudomonas sp.	0·45	0·40	0·52
P. fluorescens	0·40	0·40	0·30
P. non liquefaciens	0·40	0·40	0·32
Staphylococcus aureus	0·02	0·15	0·17

Quantitative determination of reactants and products in γ-glutamyl transfer reactions

Quantitative data on the cleavage of glutathione and on the appearance of the breakdown products, as measured by the alloxan method and quantitative paper

chromatography, are given in Table 2. In the absence of added acceptor most of the glutathione was split into glutamic acid (Glu) and cysteinylglycine (CySH-Gly), according to the reaction

$$GSH + H_2O = Glu + CySH\text{-}Gly. \qquad I$$

A new product (R_F value lower than glutathione) was detected by paper chromatography; acid hydrolysis of this compound yielded glutamic acid, cysteic acid and glycine at a ratio of 2 : 1 : 1. This product was identified as glutamylglutathione, probably γ-glutamylglutathione. The formation of this tetrapeptide may be through a transfer reaction in which glutathione served both as donor and acceptor of the γ-glutamyl moiety

$$2\ GSH = Glu\text{-}\gamma\text{-}GSH + CySH\text{-}Gly. \qquad II$$

The alloxan method does not distinguish between glutathione and γ-glutamylglutathione (Revel & Ball, 1959). Therefore the discrepancy in the amounts of glutathione cleaved as measured by the two methods (Table 2) can be explained. Glutamic acid, which is formed only in reaction I, can be taken as an index of the amount of γ-glutamyl transfer to water. The chromatographic method of analysis showed that 4 μmole of GSH disappeared; of this 2·7 μmole could be accounted for by hydrolytic cleavage (reaction I), while the rest (1·3 μmole) was used to form 0·65 μmole of γ-glutamylglutathione (reaction II). Lack of a γ-glutamylglutathione marker prevented the quantitative determination of this product. However, the amount of cysteinylglycine formed (3·3 μmole) verified the accuracy of the calculation, according to which 3·35 μmole cysteinylglycine (2·7 + 0·65) should have been formed. The γ-glutamyl transfer to glutathione (reaction II) amounted, therefore, to 19·4 % of the glutathione cleaved.

Table 2. *Glutathione breakdown in the presence and absence of acceptor*

The reaction mixtures, total volume 1·0 ml., contained: GSH, 20 μmole, or GSH, 20 μmole, and acceptor, 80 μmole; tris buffer (pH 8·5), 400 μmole; and 1·6 mg. *Proteus morganii* extract. Incubation under nitrogen for 45 min. at 37°. The disappearance of GSH was measured by the alloxan method and quantitative paper chromatography. Glutamic acid, cysteinylglycine and peptide formation were measured by quantitative paper chromatography. Data obtained by chromatography are the average of two runs with two different solvents, propanol + water and tertiary butanol + formic acid + water.

	GSH cleavage		Reaction products		
Reaction mixture	Alloxan method (μmole)	Chromatography (μmole)	Glutamic acid (μmole)	Cysteinylglycine (μmole)	γ-glutamylpeptide (O.D. × 100)
1. GSH	2·4	4·0	2·7	3·3	4·8
2. GSH + L-methionine	7·6	7·3	0	7·0	22·0
3. GSH + glycylglycine	9·9	10·5	0	10·0	20·0

In the presence of acceptors (glycylglycine or L-methionine) both methods gave identical results. Neither glutamic acid nor γ-glutamylglutathione was detected; cysteinylglycine in equivalent amount to the glutathione cleaved and a new compound were isolated. Acid hydrolysis of the new compound, formed in the presence of L-methionine, yielded equivalent amounts of glutamic acid and methionine. Hydrolysis of the DNP-derivative of this compound yielded DNP-glutamic acid and methionine. The peptide formed in the presence of glycylglycine yielded, upon

hydrolysis, glutamic acid and glycine in a ratio of 1 : 1·6. This ratio was apparently due to the presence of a dipeptidase acting on glycylglycine; glycylglycine together with glycine served as acceptors for the γ-glutamyl moiety. Hydrolysis of the DNP-derivative of this compound gave DNP-glutamic acid and glycine. The only reaction that took place in the presence of either of the acceptors tested was

$$\text{GSH} + \text{acceptor} = \text{Glu-}\gamma\text{-acceptor} + \text{CySH-Gly}. \qquad \text{III}$$

At pH 8·5 (at which the reactions were tested), neither cysteinylglycinase nor γ-glutamyllactamase activities could be detected. The former enzyme was present in the extract but showed no activity at pH 8·5.

Kinetics of γ-glutamyl transfer

The kinetics of the γ-glutamyl transfer from glutathione to amino acids, peptides and water were studied in cell-free extracts of *Proteus morganii*.

Enzyme concentration. The enzymic breakdown of glutathione in the presence or absence of acceptor was found to be proportional to the enzyme concentration (Fig. 1). At high enzyme concentrations, glutathione became limiting and the effect of the acceptor in increasing γ-glutamyl transfer was decreased. Experiments were therefore done at enzyme concentrations that cleaved 25–30% of the substrate in the absence of acceptor.

Substrate concentration. The effect of substrate concentration on enzyme activity is depicted in Fig. 2. A constant ratio of glutathione to acceptor was kept, while varying the substrate concentration.

Effect of time. The effect of time on enzyme activity in the presence of glycylglycine is shown in Fig. 3. It was measured by three different methods: disappearance of glutathione (alloxan method), formation of cysteinylglycine and synthesis of γ-glutamyl-peptide (quantitative paper chromatography). Similar progress curves were obtained with each method.

Reduced and oxidized glutathione as γ-glutamyl donors. Since both donors yielded peptides of identical chromatographic properties, the efficiency of these donors was measured by comparing the amounts of peptide formed. Oxidized glutathione (in equivalent amounts) was only 50% as active as reduced glutathione (Table 3). The higher efficiency of GSH was also found by Fodor *et al.* (1953), with a purified kidney extract.

Effect of pH value. The optimal pH value for γ-glutamyl transfer was 8·3–8·5 (Fig. 4). The pH activity curves resembled those obtained by Ball *et al.* (1956) for a kidney preparation but with some differences on the acid side of the optimal pH which will be discussed.

Effect of acceptor concentration. Both of the acceptors used increased the γ-glutamyl transfer; glycylglycine was found to be more effective than L-methionine (Fig. 5). High acceptor concentrations decreased the amount of glutathione cleaved. The inhibitory effect of high acceptor concentrations has been described in other reports (Fodor *et al.* 1953; Hird & Springell, 1954*a*; Revel & Ball, 1959). With the bacterial extract, the inhibition caused by the acceptor was competitive and could be annulled by increasing the concentration of the substrate (Table 4).

Acceptor efficiency. Data on the comparative efficiency of different amino acids and peptides, as acceptors in the γ-glutamyl transfer reaction, are given in Table 5.

Only L-amino acids were tested, since D-amino acids showed no activity. The effect of the acceptors on the γ-glutamyl transfer was assayed at two different concentrations at which neither glycylglycine nor L-methionine was inhibitory. All amino acids and peptides tested served as acceptors for the γ-glutamyl moiety; the exceptions were proline and hydroxyproline, which lack a free NH_2 group.

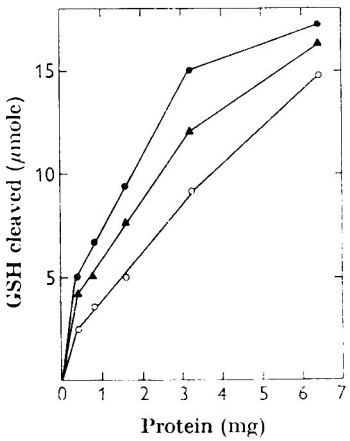

Fig. 1

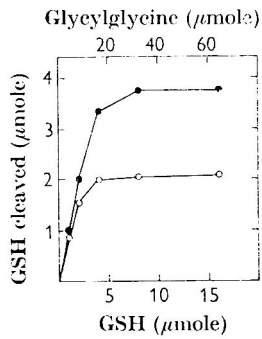

Fig. 2

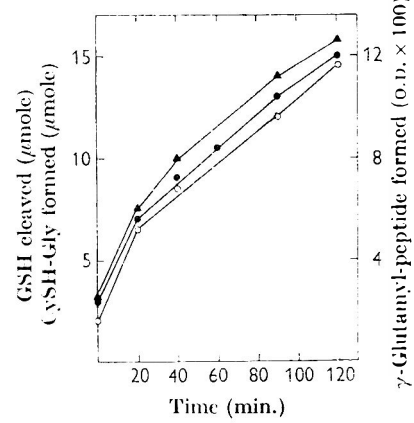

Fig. 3

Fig. 1. Effect of enzyme concentration on γ-glutamyl transfer. ○ GSH, 20 μmole; ▲, GSH 20 μmole + L-methionine 80 μmole; ●, GSH 20 μmole + glycylglycine 80 μmole. The different reaction mixtures, total volume 1·0 ml., contained, in addition to the compounds listed, tris buffer (pH 8·5) 400 μmole and *Proteus morganii* extract as indicated in the figure. Incubation under nitrogen for 45 min. at 37°. GSH cleavage was determined by the alloxan method.

Fig. 2. Effect of substrate concentration on γ-glutamyl transfer. The reaction mixtures, total volume 1·0 ml., containing GSH in various concentrations (○—○) or various concentrations of GSH + glycylglycine (●—●) were incubated for 45 min. at 37° under nitrogen. The reaction mixtures also contained tris buffer (pH 8·5), 400 μmole, and *Proteus morganii* extract 1·6 mg. protein. GSH cleavage was determined by the alloxan method.

Fig. 3. Effect of time on γ-glutamyl transfer. The experiment was performed in Thunberg tubes under nitrogen. The reaction mixtures, total volume 1·0 ml., contained GSH, 20 μmole; glycylglycine, 80 μmole; tris buffer (pH 8·5), 400 μmole; and *Proteus morganii* extract, 1·6 mg. protein. Incubation at 37°. At intervals tubes were removed and the amount of products determined. GSH cleavage (○—○) (alloxan method); cysteinylglycine formation (●—●) and γ-glutamyl peptide formation (▲—▲) (quantitative paper chromatography). Data obtained by paper chromatography are the average of two runnings using two different solvents (propanol + water and tertiary butanol + formic acid + water). Owing to the lack of γ-glutamyl peptide markers, quantitative data on peptide formation are represented as the optical density of the ninhydrin complex of the peptides at 505 mμ.

The effect of acceptor concentration on the transfer reaction is also seen in Table 5. The inhibition by the acceptor has been shown to be competitive (see Table 4). It is worthy of note, therefore that the strongest inhibition of the transfer reaction was caused by glutamine and cysteine, which resemble closely or constitute parts of the glutathione molecule.

DISCUSSION

The search for γ-glutamyl transfer activity revealed a wide distribution of the enzyme in bacteria. In mammals this activity is restricted to kidney and pancreas (Woodward, Munro & Schroeder, 1935; Binkley & Nakamura, 1948; Revel & Ball

1959). In most of the bacterial strains tested here, addition of an acceptor increased the γ-glutamyl transfer. However, in a few organisms addition of acceptor was without effect as was also found with fish kidney extract (Ball, Cooper & Clarke, 1953). Since our experiments were performed with washed organisms, the lack of acceptor effect might be attributed to permeability barriers.

The cleavage of glutathione in the absence of acceptor (at alkaline pH values) yielded less glutamic acid than could be accounted for by the amounts of glutathione split or cysteinylglycine formed. Similar results have been described with

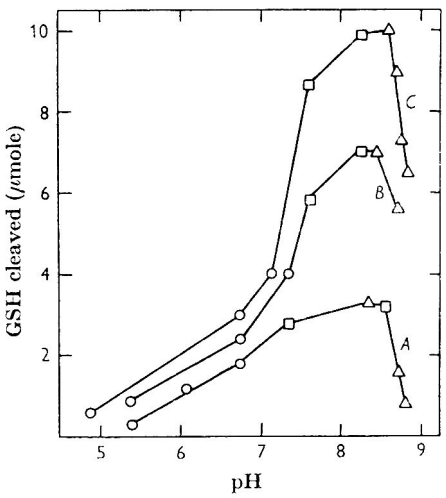

Fig. 4

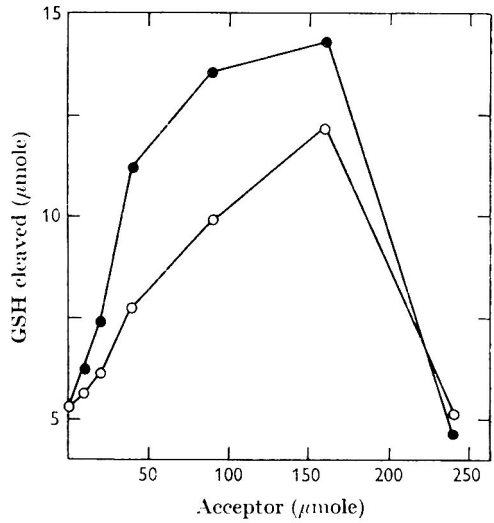

Fig. 5

Fig. 4. Effect of pH on γ-glutamyl transfer. (*A*) GSH 20 μmole; (*B*) GSH 20 μmole + L-methionine 80 μmole; (*C*) GSH 20 μmole + glycylglycine 80 μmole. The different reaction mixtures, total volume 1·0 ml., contained, in addition to the compounds listed, *Proteus morganii* extract—1·6 mg. protein—and phosphate buffer 750 μmole (○); tris buffer 400 μmole (□); or borate buffer 300 μmole (△). Incubation under nitrogen at 37° for 45 min. GSH cleavage was determined by the alloxan method. All pH values were determined at the end of incubation period with a glass electrode.

Fig. 5. Effect of acceptor concentration on γ-glutamyl transfer. GSH 20 μmole + varying amounts of L-methionine (○—○) or glycylglycine (●—●) were incubated under nitrogen for 45 min. at 37°. The different mixtures, total volume 1·0 ml., contained, in addition to the compounds listed, tris buffer (pH 8·5) 400 μmole and *Proteus morganii* extract, 1·6 mg. protein. GSH cleavage was determined by the alloxan method.

Table 3. *Comparative efficiency of GSH and GSSG as γ-glutamyl donors*

The reaction mixtures, total volume 1·0 ml., contained, in addition to the substrates listed, *Proteus morganii* extract—1·6 mg. protein—and tris buffer (pH 8·5) 400 μmole. Incubation under nitrogen for 45 min. at 37°. Peptide formation was measured by paper chromatography, using propanol water as solvent. The data were expressed as the optical density × 100 of the eluted ninhydrin–peptide complex.

Reaction mixtures	Peptide formation o.d. × 100 (at 505 mμ)
20 μmole GSH + 80 μmole L-methionine	11·0
10 μmole GSSG + 80 μmole L-methionine	5·0
20 μmole GSH + 80 μmole glycylglycine	10·0
10 μmole GSSG + 80 μmole glycylglycine	5·5

Table 4. *Effect of ratio of acceptor: GSH on γ-glutamyl transfer*

The different reaction mixtures, total volume 1·0 ml., contained in addition to the listed compounds tris buffer (pH 8·5), 400 μmole, and *Proteus morganii* extract, 1·6 mg. protein. Incubation for 45 min. at 37° under nitrogen. GSH cleavage was determined by the alloxan method. In the last column GSH cleavage is expressed as a percentage of the amount of GSH cleaved in the absence of added acceptor.

Reaction mixture				GSH cleaved	
GSH (μmole)	Glycylglycine (μmole)	L-methionine (μmole)	Acceptor: GSH	μmole	%
20	—	—	—	5·3	100
20	240	—	12	5·2	98
20	—	240	12	5·1	96
20	120	—	6	15·4	290
20	—	120	6	10·8	205
40	—	—	—	5·4	100
40	240	—	6	16·5	306
40	—	240	6	11·5	214

Table 5. *Efficiency of various amino acids and peptides in promoting γ-glutamyl transfer*

The different reaction mixtures, total volume 1·0 ml., contained GSH, 20 μmole; acceptor as indicated in the table; tris buffer (pH 8·5), 400 μmole; and *Proteus morganii* extract, 1·6 mg. protein. Incubation 45 min. at 37° under nitrogen. The data, which were obtained by the alloxan method, are expressed as the percentage of γ-glutamyl transfer as effected by the concentration of the corresponding acceptor.

Acceptor added	γ-glutamyl transfer			Amino pK*
	40 μmole	80 μmole	160 μmole	
None	100	100	—	—
L-Alanine	116	106	—	9·87
L-Arginine	103	127	—	8·99
L-Asparagine	125	151	—	8·87
L-Cysteine	150	65	—	8.36
L-Glutamic acid	163	124	—	9·96
L-Glutamine	165	95	—	9·13
Glycine	116	151	—	9·78
L-Histidine	194	186	—	9·17
L-Hydroxyproline	101	101	—	9·62
L-Leucine	116	111	—	9·74
L-Lysine	141	117	—	9·18
L-Methionine	182	196	—	9·21
L-Phenylalanine	230	240	—	9·13
L-Proline	90	85	—	10·64
L-Serine	169	120	—	9·21
L-Threonine	128	142	—	9·10
L-Tyrosine	128	—	—	9·11
L-Valine	169	125	—	9·72
DL-Alanyl-DL-alanine	—	200	204	8·42
DL-Alanylglycine	—	207	204	8·18
Glycylglycine	213	222	—	8·25
Glycyl-L-methionine	213	200	—	—
Glycylglycylglycine	157	164	—	7·91

* Amino pK values from *Biochemist's Handbook* (C. Long, editor), 1961, pages 43–52, E. & F. N. & Son Ltd. London.

kidney extracts (Woodward & Reinhart, 1942; Fodor *et al.*, 1953; Revel & Ball, 1959). In some of these cases the lack of stoichiometry was accounted for by pyrrolidone carboxylic acid formation (Woodward & Reinhart, 1942; Fodor *et al.* 1953), probably through γ-glutamyl lactamase activity (Connel & Hanes, 1956), but no pyrrolidone carboxylic acid formation was detected with the *Proteus morganii* extract. On the other hand, glutathione served both as acceptor and as donor of the γ-glutamyl residue. Transfer of the γ-glutamyl residue to glutathione has not been reported previously. The possibility of such a reaction was discussed by Revel & Ball (1959), who did not, however, obtain any evidence for such a reaction product. In the presence of acceptor the transfer reaction yielded equivalent amounts of γ-glutamyl-acceptor and cysteinylglycine. These results are in agreement with those obtained with kidney extracts (Revel & Ball, 1959).

The kinetic data obtained with the *Proteus morganii* extract are similar to those obtained with kidney extracts (Fodor *et al.* 1953; Hird & Springell, 1954*a*; Ball *et al.* 1956; Revel & Ball, 1959). However, some differences were encountered regarding the correlation between acceptor ability and pK value. It is assumed that the acceptor acts in its unprotonated form (Johnston, Mycek & Fruton, 1950; Fruton, Johnston & Fried, 1951; Revel & Ball, 1959; Avi Dor, 1960). If this is correct, acceptor activity should be inversely proportional to the pK value of the amino group. Comparison of acceptor activity and pK value (Table 5) shows that this was generally the case with the bacterial extract. However, there were a few exceptions to this rule; e.g. glycylglycylglycine, with a pK value lowest of all acceptors tested (7·91), showed poor acceptor ability. It seems, therefore, that factors other than the pK value may influence the suitability of the acceptor. Similar findings were reported also with kidney extracts (Fodor *et al.* 1953). On the other hand, there were some differences between the *Proteus morganii* enzyme and the kidney extract in regard to the efficiency of certain acceptors (Fodor *et al.* 1953; Ball *et al.* 1956). Thus, glutamine (pK 9·13), which was one of the best acceptors for the kidney extract, showed moderate acceptor activity similar to that of glutamic acid (pK 9·96). Dipeptides, although exhibiting better acceptor capacity than amino acids in the bacterial system, were considerably less active there than in the kidney system. And, most important, lower pH values should have increased the acceptor effectivity of dipeptides as compared with amino acids. However, no such increase in the relative activities at acid pH values was encountered, when acceptor abilities of glycylglycine and of L-methionine were compared using the bacterial extract (Fig. 4).

The above-mentioned discrepancies can be explained by the presence of glutaminase and peptidase activities in the bacterial extract. The rapid deamination of glutamine resulted in the formation of a less active acceptor—glutamic acid. The partial degradation of peptides, by peptidases present in the crude extract, decreased the apparent acceptor ability of the dipeptide.

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Antigenic variation in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis*

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SUMMARY

Antigenic variation was studied in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis*. In goats and rabbits infected by tsetse flies, the antigenic character of the strain did not change until the 7th day of infection; thereafter new antigens developed at 2- to 3- day intervals until the infected animal died. The antigens of the *T. brucei* strain developed in a similar sequence in the early stages of infections induced by different tsetse flies; in later stages of the infections, the sequences in which antigens developed varied, but many of those produced in different hosts were similar. A common antigen, provisionally called the basic strain antigen, occurred in all substrains of the strain isolated during the first 7 days of infection from animals infected by different tsetse flies. This basic strain antigen was relatively stable and, when present in trypanosomes ingested by tsetse flies, it persisted throughout the period required for cyclical development and for the remainder of the life of the infective fly. It also tended to displace variant antigens when trypanosomes with such antigens multiplied in environments free from antibody. Tsetse flies which ingested trypanosomes with variant antigens transmitted trypanosomes with either the basic strain antigen only or with a mixture of the ingested variant antigen and the basic strain antigen. The basic strain antigen also developed at an early stage of infection when non-immune animals were infected with variants of the strain transmitted by syringe. These findings are discussed in relation to the serological classification of *brucei* subgroup trypanosomes and the immunization of animals against trypanosomiasis.

INTRODUCTION

Antigenic variation in trypanosomes was probably first described by Franke (1905) who found that the serological characteristics of a strain of *Trypanosoma equinum* changed in an infected host. Subsequent immunological studies (reviewed by Taliaferro (1930) and Soltys (1963)) have confirmed that trypanosomes have a great capacity for antigenic variation, and that both natural strains and clones can produce many different antigens. Certain aspects of antigenic variation in trypanosomes have been studied in considerable detail because of their importance in relation to the development of drug-relapse strains, the immunization of animals against trypanosomiasis and the serological classification of strains. Much has been learned, for example, about the number of antigens which may be produced by a trypanosome strain (Ritz, 1914, 1916; Lourie & O'Connor, 1937; Osaki, 1959), the

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variability of a single stock of a strain in different hosts (Leupold, 1928; Raffel, 1934; Cantrell, 1958; Inoki, 1960), the antigenic stability of relapse strains (Mesnil & Brimont, 1909; Rosenthal, 1913) and the relation between antigenic variation and the immune response of vertebrate hosts (Massaglia, 1907; Levaditi & McIntosh, 1910; Mutermilch & Salamon, 1928; Russell, 1936; Gray, 1962). More recently attempts have been made to characterize variable trypanosomal antigens and to locate their position in the organism (Weitz, 1960; Brown & Williamson, 1962, 1964; Seed, 1963; Williamson & Brown, 1964).

Much of this work on antigenic variation in trypanosomes was done with strains maintained by blood-passage in laboratory animals and comparatively little has been done to relate the findings to antigenic variation in strains transmitted by tsetse flies. The antigenic changes which occur in blood-passaged strains probably resemble those that occur in strains transmitted in the field by the interrupted feeding of biting flies of such genera as *Tabanus* and *Stomoxys*. Transmission of a trypanosome strain by a tsetse fly, in contrast to mechanical transmission, is effected only after the trypanosomes have completed a cycle of development in the fly, which usually takes at least 12 days, depending on climatic factors and on the species of trypanosome and fly (see Buxton, 1955). The importance of occasional development in tsetse flies in preserving trypanosome strain characteristics such as morphology, pathogenicity and drug-sensitivity has been recognized for many years (Bruce, 1914; Lester, 1932; Murgatroyd & Yorke, 1937) and it is surprising that the effects of cyclical development on the antigenic characteristics of trypanosomes have been, with one notable exception, largely neglected. Broom & Brown (1940) studied the serological characters of three strains of *Trypanosoma brucei* before and after cyclical development in *Glossina morsitans* and found that serological differences between variants of each strain diminished during development in the flies. Substrains of one strain transmitted by different tsetse flies resembled each other closely, but differed from cyclically transmitted substrains of other strains of the same species.

The work described in the present paper was done to extend the findings of Broom & Brown because the reduction of serological variants of a strain during development in tsetse flies to an antigenic type which might be characteristic of the strain might be of practical value in the serological classification of strains and in immunization against trypanosomiasis. The experiments were concerned with antigenic variation in a strain of *Trypanosoma brucei* in animals infected by tsetse flies, and with the effects of cyclical transmission on the antigenic characteristics of substrains and serological variants of the strain.

METHODS

The strain of Trypanosoma brucei used. The strain was isolated in 1961 from a sheep infected by *Glossina morsitans* which had been collected near Mokwa, Niger Province, Northern Nigeria. A line of the strain was serially passaged by syringe through fourteen rats before a stock was frozen and stored at $-80°$ for use in the present experiments. The strain was polymorphic when recovered from the frozen stock and transmissible by tsetse flies. Rats and mice infected by the injection of such material had acute infections with massive parasitaemias and died after 5–7 days and 3–4 days, respectively. Rats infected with the strain by tsetse flies had

acute infections and died after 8–10 days, but rabbits and goats had chronic infections with low-grade parasitaemias and died after 4–6 weeks. The period between the bite of an infective fly and the appearance of trypanosomes in the peripheral blood of rats, rabbits, goats and sheep was relatively constant, between 2 and 4 days.

Maintenance of the strain. During experiments, substrains and variants of the trypanosome were maintained for short periods by syringe passage at intervals of 2–3 days in mice. Repeated sub-passage was kept to a minimum and was only used when isolating substrains, when preparing antigens for agglutination tests and when frozen stocks of the strain were being re-established in experimental animals. Stocks of substrains and variants were maintained for longer periods at $-80°$ as were antigens for agglutination tests which were prepared as described below.

Experimental animals. White mice (12–20 g.) were used during the isolation and short-term passage of substrains and when preparing antigens for agglutination tests. Rabbits (1–2 kg.), white rats (100–200 g.) and goats and sheep of indigenous West African varieties were used to produce agglutinating antisera, to feed tsetse flies, and in experiments on antigenic variation. Mice, rats and rabbits were drawn from closed laboratory colonies and it was assumed that they were free from trypanosomal infections before experiments were started. Goats and sheep were bought in an area which is free from trypanosomiasis and they were unlikely to be harbouring trypanosomes; however, as a precaution, a wet blood film from each animal was examined daily for 30 days before the animal was used, and samples of blood were inoculated into rats which were then examined daily for trypanosomes for 4 weeks. No infections were ever detected.

Terms used to describe populations of trypanosomes. Several terms were used to describe populations of trypanosomes during the experiments. The *strain* was a collective term and included all the trypanosomes derived from the population of *Trypanosoma brucei* isolated from the sheep originally infected by wild-caught *Glossina morsitans*. The term *original strain* was used to describe the population of the strain preserved at $-80°$ at the beginning of the experiments. *Substrains* were populations of trypanosomes derived from the original strain or each other; *ingested substrains* consisted of trypanosomes ingested by tsetse flies when they fed on infected animals; *cyclically transmitted substrains* consisted of trypanosomes isolated from animals between 3 and 7 days after they were bitten by infective tsetse flies. *Serological variants* (or simply *variants*) were substrains shown by serological methods to be antigenically different from other substrains. (Variants in this sense were often called relapse strains in the earlier literature on antigenic variation; since many of the infections studied in the present experiments were not characterized by distinct crises and relapses, although antigenic variation occurred, the term relapse strain has been avoided.)

Methods of counting trypanosomes. For routine purposes, parasitaemias in infected animals were estimated by counting the trypanosomes in sixty microscope fields ($\times 40$ objective and $\times 8$ eye-piece) of a wet blood film. When accurate estimations of the numbers of trypanosomes in heavy suspensions of organisms were required, a sample of the infected material was suitably diluted with Hayem's solution (Whitby & Britton, 1953) and the organisms counted in a haemocytometer.

Infection of tsetse flies with the trypanosome

Origin and infection of the tsetse flies. Most of the tsetse flies were obtained from *Glossina* pupae collected in parts of Zaria Province, Northern Nigeria, during the dry season between December and March each year; a few flies were obtained from pupae produced in a laboratory colony of *Glossina palpalis*. The pupae were incubated in a moist atmosphere at 27–29° and the flies which emerged were kept individually in glass specimen tubes (3 × 1 in.), or in groups of twenty of the same sex in wooden Bruce boxes, in a humid atmosphere in a fly-room maintained at 24–26°.

Flies were infected by feeding on infected animals on the 1st or 2nd day after they emerged from puparia, since higher infection rates with 'brucei' subgroup trypanosomes are obtained in this way than by feeding flies later in life (Wijers, 1958). In some instances, flies were fed on infected animals for 7–14 days, but in experiments in which the antigens of trypanosomes ingested by individual flies were being compared with the antigens of the trypanosomes which the flies transmitted, they were fed on infected animals once only. After taking infected feeds, flies were maintained on uninfected rats, goats, or sheep until they became infective, were killed or died.

Isolation of infective tsetse flies. Two methods were used to isolate infective tsetse flies from groups of flies which had taken infected feeds.

(*a*) *Infection of a susceptible animal.* Towards the end of the period required for cyclical development of the trypanosome (16–18 days after an infected feed), large groups of flies were divided into subgroups of three, four or five individuals which were fed daily on a rat until it became infected. The flies in subgroups which caused infections were then fed on separate rats to identify the infective fly.

(*b*) *Examination of tsetse-fly saliva.* This method was occasionally used to find an infective fly in a small group. Hungry flies were induced to attempt to feed on a guinea-pig and a small amount of tsetse-fly saliva was collected by inserting a microscope slide carefully between the proboscis of the fly and the guinea-pig (Burtt, 1946). The saliva was dried in air, fixed with methanol and stained with Giemsa's stain, and examined microscopically for metacyclic trypanosomes.

The number of infective flies isolated. Tsetse flies are difficult to infect with 'brucei' subgroup trypanosomes and the infection rates achieved in the laboratory are usually low. Attempts were made to infect 527 *Glossina morsitans* and 510 *G. palpalis* with the strain; 18 infective *G. morsitans* and 3 infective *G. palpalis* were isolated. The period required for cyclical development of the trypanosome in *G. morsitans* averaged about 22 days, but varied from 19 to 25 days; in *G. palpalis* the period varied from 29 to 42 days.

Determination of the antigenic relationships of substrains of the trypanosome

The antigenic relations of substrains and variants of the trypanosome were determined mainly by agglutination tests. In certain instances, indications of antigenic relations from direct agglutination tests were confirmed by absorption tests.

Antigens for agglutination tests. Parasitaemias in infected rabbits and goats were usually scanty, but heavy suspensions of trypanosomes were needed for use as antigens in agglutination tests. When antigens were required, trypanosome substrains were isolated from infected animals by injecting 0·5 ml. volumes of blood

into mice, and sub-passaged from 2 to 5 times at 2 to 3-day intervals in a series of mice until they caused massive parasitaemias after only 2–3 days of infection. The antigenic characteristics of substrains did not vary when they were passaged in this was provided that the interval between successive sub-passages did not exceed 3–4 days and that the procedure was not repeated too many times. When the substrains were satisfactorily adapted to mice, antigens for agglutination tests were prepared as described by Cunningham & Vickerman (1962) and stored at $-80°$ (Polge & Soltys, 1957).

Agglutinating antisera. Rabbits and goats infected by tsetse flies produced antibodies which agglutinated trypanosomes. Sera were obtained from such animals at intervals which varied in different experiments, but which were usually between 1 and 3 days. When an antiserum to a particular substrain was needed, a suspension of washed living trypanosomes in Alsever's solution was prepared from blood of infected mice. A rabbit was given a single intravenous injection of 2·0 ml. of the suspension (containing $4–8 \times 10^8$ trypanosomes) and bled 6 days later. Such 6th-day antisera had high titres of agglutinins to the antigens of the injected substrains but did not agglutinate other antigens of the strain which developed later as a result of antigenic variation. Antisera were stored at $-20°$ until they were required.

Agglutination tests. Agglutination tests were done as described by Cunningham & Vickerman (1962). The results were read microscopically ($\times 10$ objective and $\times 10$ eye-piece) 25–30 min. after the addition of antigen to diluted antisera. The last dilution of an antiserum which produced a few discrete clumps of trypanosomes was taken as the end-point of the reaction. Good agreement was found between the results of agglutination tests made on different occasions with the same antigen + antibody system and, in general, titrations were repeated once. The titres of agglutinins in antisera are expressed as reciprocals of serum dilutions in all tables showing results of agglutination tests.

Sera were taken from all animals before they were used in experiments and tested for agglutinins to various antigens of the trypanosome strain, but in all cases such pre-infection sera failed to agglutinate trypanosomes.

Absorption tests. Suspensions of trypanosomes with which to absorb antisera were prepared as follows. Groups of rats were infected with appropriate substrains and, 2–3 days later, when the blood contained many trypanosomes, they were anaesthetized and bled out into Alsever's solution. The trypanosomes were separated from the erythrocytes by centrifugation, washed twice with Alsever's solution and resuspended in the same medium. Suspensions were prepared in this way to contain $8–14 \times 10^8$ trypanosomes/ml.

To absorb agglutinins from an antiserum, 0·7 ml. of trypsanosome suspension was mixed with 0·1 ml. of antiserum in a centrifuge tube and left on the bench for 1 hr. The tube was then centrifuged at 3000 rev./min. for 10 min. with an M.S.E. 'Major' centrifuge (Measuring & Scientific Equipment, London) and 0·2 ml. of the clear supernatant fluid removed and mixed with 1·8 ml. of trypanosome suspension in another centrifuge tube. After one further hour on the bench, the tube was centrifuged at 3000 rev./min. for 10 min. and the supernatant fluid separated and placed in a clean tube. A sample of the same antiserum was diluted with saline for control purposes. When testing absorbed sera for residual agglutinins, it was estimated that they had been diluted 1/80 during the absorption procedures.

RESULTS

Antigenic variation in the strain of Trypanosoma brucei in animals infected by tsetse flies

A rabbit and two goats were infected with the trypanosome by single bites of isolated tsetse flies to determine the period between the bite of a fly and the appearance of trypanosomes and antibodies in the blood, and the course of antigenic

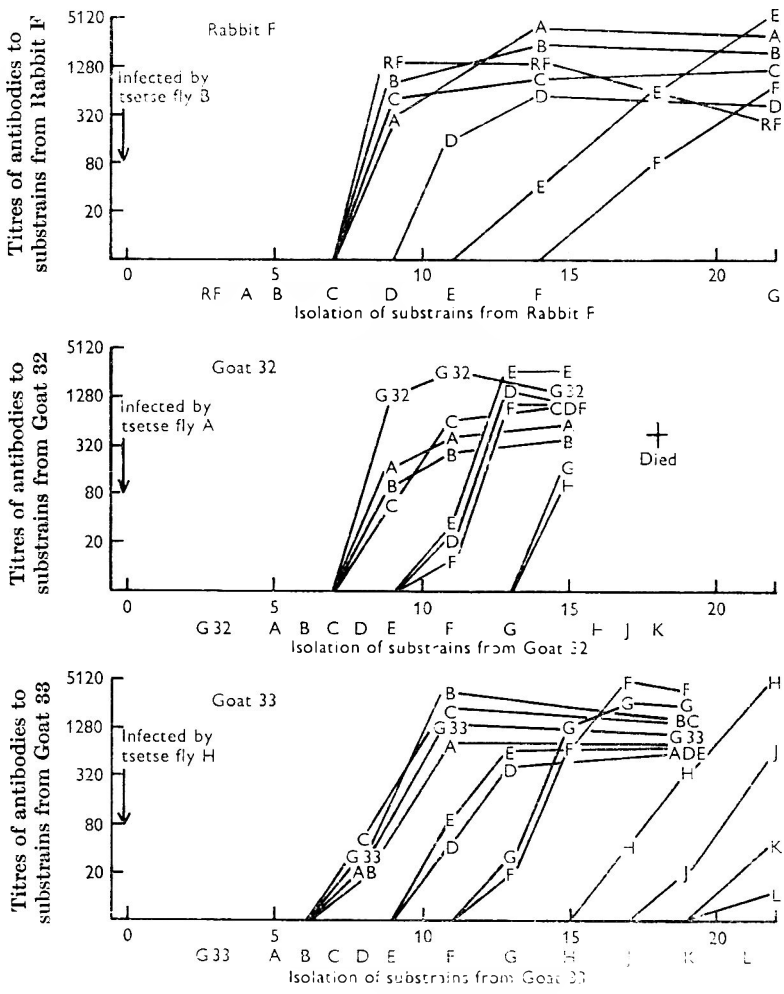

Fig. 1. Antigenic variation in *Trypanosoma brucei* in animals infected by tsetse flies as shown by agglutinin production to substrains isolated from each animal; similarity of antibody production to groups of substrains indicates their antigenic similarity. A–A, B–B, etc., production of agglutinins to substrains A, B, etc.

variation in the trypanosomes. Substrains of trypanosomes were isolated during the infections at intervals which varied from 1 to 3 days. The first substrain isolated from each animal was identified by the number of the animal and those isolated

subsequently were lettered in sequence. For example, the substrains isolated from Rabbit F were RF, RFA, RFB, RFC and so on to RFG while those from Goat 32 were designated G 32, G 32A, G 32B, G 32C,...to G 32K. Sera were obtained from the animals before infection and afterwards at 2- to 3-day intervals and tested for agglutinins to antigens prepared from substrains isolated from the same animal.

Sub-inoculation of small volumes of blood into mice showed that trypanosomes were present in the blood of the goats and the rabbit on the 3rd day after the bite of an infective tsetse fly, and agglutination tests showed that antibodies appeared in the sera of these animals on the 8th day of infection (Fig. 1). The antigenic character of the infecting trypanosomes did not change in any instance until the 7th day of infection because serum taken from each animal on day 9 agglutinated substrains isolated between day 3 and day 7 but not those isolated later. New antigens of the trypanosome strain developed between day 7 and day 9 and the first serologically distinct substrains were isolated from both goats on day 8 and from the rabbit on day 9; thereafter, substrains with different serological characters were isolated at about 3-day intervals until the animals died.

Reactions between sera and trypanosome substrains indicated that five serological variants were isolated from Rabbit F (represented in experiments described below by substrains RFA, RFD, RFE, RFF and RFG); similarly, four variants were isolated from Goat 32 (represented subsequently by substrains G 32A, G 32D, G 32G, G 32K) and probably 11 variants were isolated from Goat 33 during an infection which lasted for 30 days.

A comparison of antigens which developed in animals infected by different tsetse flies

The relationships of the antigens of the trypanosome strain which developed in animals infected by different tsetse flies were investigated as follows. Sera were collected at 2–3 day intervals from three goats which had been infected by different tsetse flies, and were tested for agglutinins to five variants of the strain isolated in the previous experiment from Rabbit F.

The antigens which developed during the four infections were similar, for sera from the goats agglutinated the rabbit variants (Fig. 2). During the early stages of the infections the antigens tended to develop in a similar sequence. The first trypanosomes which appeared in the blood of the four goats were antigenically similar, because the agglutinins produced by each goat after 9 days of infection reacted strongly with the first rabbit variant (variant RFA); 3–4 days later, each goat produced agglutinins to variant RFD, while after a further 3–6 days two of the goats had produced agglutinins to variant RFE (the other goat was dead). Sera taken from the surviving goats 18–24 days after they were bitten by tsetse flies also agglutinated trypanosome variants RFF and RFG.

Although antigens related to those which developed during the rabbit infection also developed during the goat infections, they were probably not the only antigens which developed in the goats. For example, the homologous reactions between sera and variants from Goat 32 indicated that the goat produced antibodies to three variants of the strain before it died (see Fig. 1), but the same goat sera agglutinated only two of the five rabbit variants (Fig. 2).

These findings were confirmed when the experiment was repeated a year later with variants and sera from a different group of animals consisting of three rabbits

and two goats. Similar antigens developed during the five infections and again, in the early stages of infection, they developed in a similar sequence. However, there was more variation of the period between the bites of infective flies and the first

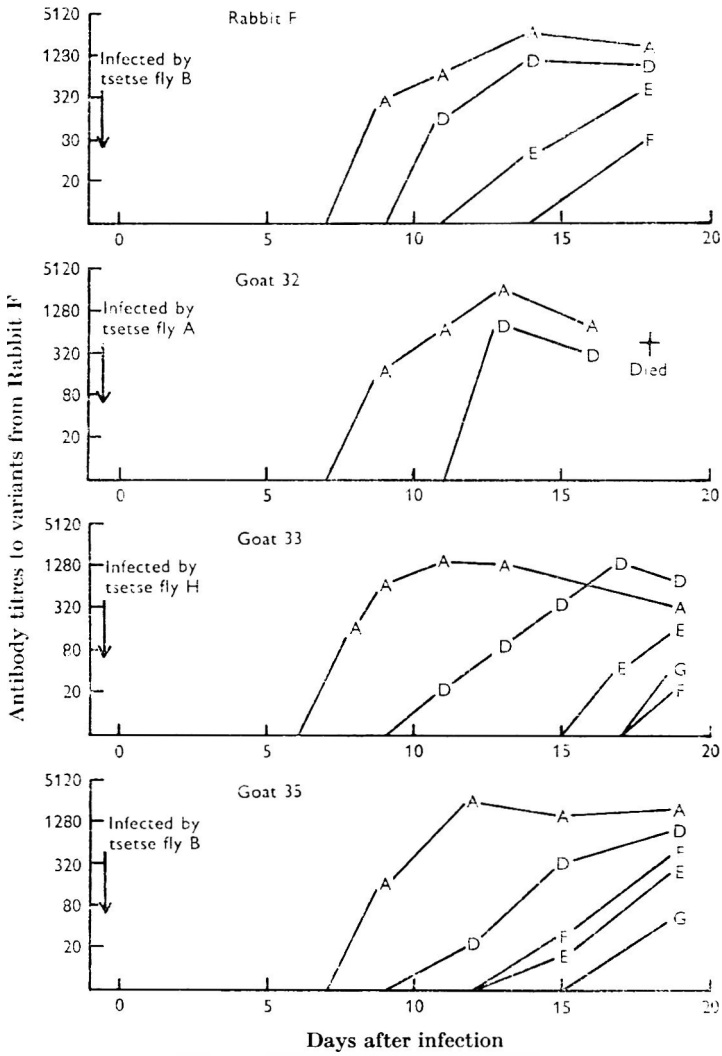

Fig. 2. Comparison of agglutinin production by three goats infected with *Trypanosoma brucei* by different tsetse flies to five variants of the strain isolated from an infected rabbit, showing that similar antigens develop during different infections. A–A, D–D, E–E, F–F and G–G, production of agglutinins to variants A, D, E, F and G, respectively.

appearance of antibody in the serum of the animals in this experiment, and the sequences in which the antigens of the strain developed during the later stages of the infections, as shown by the sequences in which antibodies were produced, also varied considerably. Figure 3 shows the variation of the time taken by individual animals to produce agglutinins to a titre of 1/160 to each of the variants isolated from the reference animals in the two experiments.

The antigenic similarity of substrains transmitted by different tsetse flies

The results of the preceding experiments suggested that the trypanosomes transmitted by different tsetse flies were antigenically similar. These preliminary observations were extended as follows.

Cyclically transmitted substrains were collected from twenty-one animals which had been infected by different tsetse flies; each substrain was isolated within the first 3–4 days of an infection, before antigenic variation occurred, and therefore probably consisted of trypanosomes of the antigenic type transmitted by the tsetse fly.

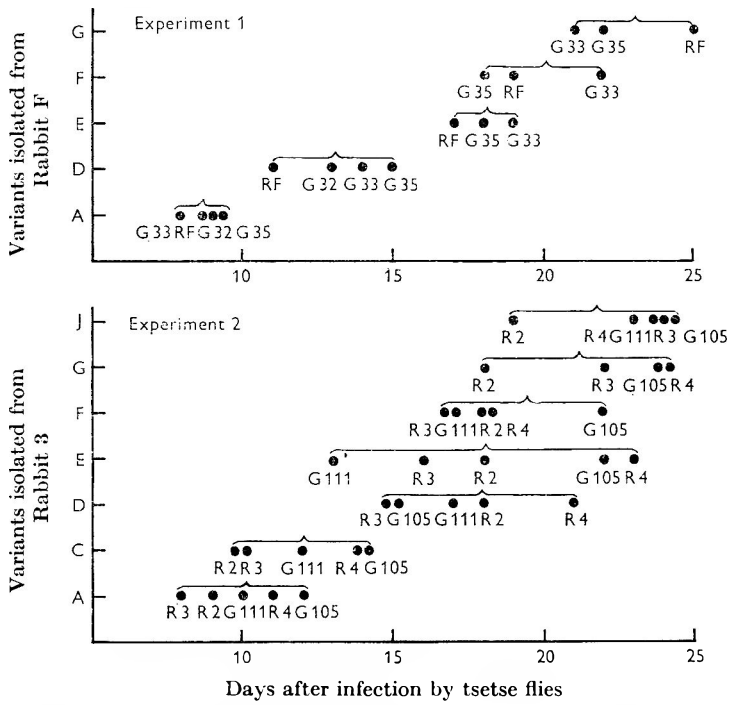

Fig. 3. The sequences in which antigens of *Trypanosoma brucei* developed in animals infected by different tsetse flies. Variation of the periods which elapsed between infection and agglutinin production to a titre of 1/160 by a rabbit (RF) and three goats (G32, G33, G35) to five variants isolated from the rabbit RF (Experiment 1) compared with variation of agglutinin production by another group of three rabbits (R2, R3, R4) and two goats (G105, G111) to variants of the same strain isolated a year later from a different rabbit (R3) (Experiment 2).

Owing to the seasonal availability of tsetse flies, eight of the cyclically transmitted substrains were isolated in 1962 and thirteen in 1963; as the methods used to compare the two groups of substrains differed slightly, the results of the experiments are presented in two sections.

Comparison of substrains transmitted in 1962. The antigenic relationships of the substrains transmitted in 1962 were determined by direct agglutination tests; a 6th-day antiserum to each substrain was tested for agglutinins to the homologous substrain and to some or all of the other substrains in the group. The substrains

had antigens in common since, with one exception, an antiserum which had a high titre of agglutinins to its homologous substrain also agglutinated the other substrains. The antiserum to the substrain from Goat 33, which had a high titre of agglutinins to seven of the substrains, was exceptional in that it did not agglutinate the substrain from Rat 4 (Table 1).

Table 1. *Titres of agglutinins in antisera to substrains of Trypanosoma brucei transmitted by tsetse flies in 1962*

Tsetse fly which transmitted the substrain	Substrain used as antigen	Titres of agglutinins in antisera to substrain							
		Rat 1	Rat 2	G28	G29	Rat 4	Rat 5	Rat 6	G33
A	Rat 1	1280	1280	5120	160	640	320	320	640
B	Rat 2	5120	2560	5120	2560	1280	2560	2560	640
C	G28	320	320	2560	640	320	1280	640	5120
D	G29	640	640	5120	5120	640	320	1280	320
E	Rat 4	.	.	.	.	640	80	160	–
F	Rat 5	.	.	.	.	320	1280	640	1280
G	Rat 6	.	.	.	.	2560	2560	2560	1280
H	G33	.	.	.	.	320	640	320	2560

– = no agglutination; . = not tested

Table 2. *Titres of agglutinins in rabbit antisera to antigens prepared from a substrain of Trypanosoma brucei transmitted by a tsetse fly in 1962 (the reference substrain) and from substrains transmitted by tsetse flies in 1963*

Tsetse fly which transmitted the substrain	Substrain used as antigen	Titres of agglutinins in an antiserum to the reference substrain	Titres of agglutinins in antisera to the 1963 substrains	
			(*a*) To the homologous substrain	(*b*) To the reference substrain
B	The reference substrain	2560	na	na
1	Rat 101	160	1280	2560
2	Rat 105	80	1280	320
3	Rat 109	2560	1280	640
4	Rat 113	5120	5120	5120
5	Rat 117	5120	5120	5120
6	Rat 121	2560	2560	1280
8	Rat 301	1280	2560	2560
7	Rat 302	2560	2560	2560
9	Rat 303	1280	640	1280
10	Rat 304	2560	640	2560
11	Rat 305	5120	2560	2560
12	Rat 306	5120	2560	2560
13	Rat 203	5120	5120	5120

na = not applicable.

Comparison of trypanosome substrains transmitted in 1963. The relationships of the substrains transmitted in 1963 were determined to one of the cyclically transmitted substrains isolated in 1962, first by direct agglutination tests and then by absorption experiments. The substrain isolated from Rat 2 (see Table 1), which had been stored for a year at −80°, was used as a reference substrain for these purposes. A 6th-day antiserum with a high titre of agglutinins to the reference substrain had a similar high titre to eleven, and a lower titre to two, of the 1963 substrains, indicating

that they all had antigens in common. This finding was confirmed by tests with antisera to each of the 1963 substrains, which all agglutinated the reference substrain (Table 2). Antisera to each of the 1963 substrains were then absorbed with a suspension of the reference substrain and the absorbed samples were tested for residual agglutinins to their homologous substrains and to the reference substrain. The absorption tests again confirmed that the 1963 substrains and the reference substrain had an antigen in common and showed that at least three of the 1963 substrains (from Rats 203, 301, 302) possessed an additional antigen because antisera to these substrains still agglutinated their homologous antigens after agglutinins to the reference substrain had been absorbed (Table 3). The antigen which occurred in all the cyclically transmitted substrains isolated in 1963 and in those isolated in 1962, represented by the substrain from Rat 2, was provisionally named the *basic strain antigen* for the purposes of the following experiments.

Table 3. *Effects of absorbing antisera to substrains of Trypanosoma brucei transmitted by tsetse flies in 1963 with a reference substrain transmitted by a tsetse fly in 1962*

Substrain used as antigen	Titres of agglutinins in antisera diluted with saline to the		Titres of agglutinins in antisera absorbed with the reference substrain to	
	Homologous substrain	Reference substrain	Homologous substrain	Reference substrain
101	320	2560	–	–
105	1280	1280	–	–
109	2560	5120	–	–
113	5120	5120	–	–
305	1280	2560	–	–
117	5120	5120	–	–
121	640	1280	–	–
203	2560	2560	2560	–
301	2560	5120	2560	–
302	1280	2560	640	–
303	320	640	–	–
304	2560	2560	–	–
306	2560	5120	–	–

– = no agglutination.

The effect of development in the tsetse fly on the antigens of substrains

The antigenic similarity of the cyclically transmitted trypanosome substrains might have been due either to the ingestion of trypanosomes with the basic strain antigen by all the tsetse flies, to alterations of the antigens of serological variants during cyclical development, or to both factors. To investigate these possibilities, attempts were made to infect some tsetse flies with trypanosomes with the basic strain antigen and others with trypanosomes with variant antigens, and to compare the antigens of ingested trypanosomes with those of the organisms the tsetse flies transmitted.

To obtain the material required for these purposes, several batches of 20–30 newly emerged tsetse flies were each fed once on a reservoir animal infected either with one of several serological variants of the trypanosome strain or with a substrain with the basic strain antigen. To guard against the possibility of antigenic

Table 4. *The effects of cyclical development on trypanosomes with the basic antigen and variant antigens of a strain of Trypanosoma brucei; titres of agglutinins in antisera to a reference substrain with the basic strain antigen and to substrains ingested and transmitted by individual tsetse flies*

Group of tsetse flies		Tsetse flies which transmitted the substrains	Reactions of antisera to reference substrain with antigens prepared from			Reactions of antisera to ingested substrain with antigens prepared from			Reactions of antisera to transmitted substrain with antigens prepared from		
			Reference substrain	Ingested substrain	Transmitted substrain	Reference substrain	Ingested substrain	Transmitted substrain	Reference substrain	Ingested substrain	Transmitted substrain
Part 1. Flies which ingested substrains with the basic strain antigen		F	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		H	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		4	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		5	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		7	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		8	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		9	+ + +	+ + +	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +
		10	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+ + +	+ +	| | |
		12	+ + +	+ + +	+ + +	+	+ + +	+ + +	+ + +	+ +	+ + +
Part 2. Flies which ingested substrains with variant antigens	(*a*)	E	+ + +	−	+ +	+	+ + +	−	+ + +	−	+ + +
		G	+ + +	−	+ + +	+	+ +	+	+ + +	−	+ + +
	(*b*)	1	+ + +	−	+ +	−	+ + +	−	+ + +	| | |	| | |
		2	+ + +	−	+ +		+ + +	−	+ +	+ +	+ + +
		3	+ + +	−	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +
		6	+ + +	−	+ + +	−	+ + +	−	+ + +	+ + +	+ + +
		11	+ + +	−	+ + +	−	+ + +	−	+ + +	+ + +	+ + +
		13	+ + +	−	+ + +	−	+ + +	−	+ + +	+ + +	+ + +

−, No agglutination; +, titre of 1/10 to 1/40; + +, titre of 1/80 to 1/320; + + +, titre above 1/640.

variation in the trypanosomes in the reservoir animals before the flies were fed, an ingested substrain of the antigenic type taken by each batch of flies was isolated from the reservoir host while the flies were being fed, and preserved at −80°. Flies which had taken infected feeds were maintained on uninfected hosts for about 3 weeks while the trypanosomes completed their cycle of development. An infective tsetse fly was then isolated from each batch of flies and used to infect a rat or a goat from which a substrain of the antigenic type transmitted by the fly was subsequently isolated. Antigens for agglutination tests were prepared from the substrains and 6th-day agglutinating antisera were prepared in rabbits. The antigenic relationships of the substrains ingested and transmitted by each tsetse fly were then determined by agglutination tests to each other and to a reference substrain (the substrain from Rat 2) known to possess the basic strain antigen.

The effect of cyclical development on trypanosomes with the basic strain antigen. Nine tsetse flies which had ingested trypanosome substrains with the basic strain antigen became infective. In these instances the ingested substrains were serologically indistinguishable from the reference substrain so that an antiserum to either the reference or the ingested substrain had a similar titre of agglutinins to both substrains. The basic strain antigen persisted during cyclical development in these flies because each cyclically transmitted substrain was agglutinated by antisera to the reference and to the relevant ingested substrain, and an antiserum to each cyclically transmitted substrain had a similar titre to the homologous antigen, the reference substrain and the relevant ingested substrain (Table 4, Part 1).

The effect of cyclical development on trypanosomes with variant antigens. Eight tsetse flies which had ingested serological variants of the trypanosome strain became infective; in these instances the ingested substrains were not agglutinated by the antiserum to the reference substrain. This finding was supported by the reactions of antisera to the ingested substrains, five of which agglutinated only the homologous substrain while the other three had high titres to the homologous substrain and low titres to the reference and cyclically transmitted substrains (Table 4, Part 2).

Two of these tsetse flies, Flies E and G, transmitted trypanosome substrains which were closely related to the reference substrain and unrelated to the substrains which they ingested. The variant antigens of substrains ingested by these flies were apparently completely replaced by the basic strain antigen because in each case an antiserum to the cyclically transmitted substrain, which had high titres to the homologous and reference substrains, did not agglutinate the ingested substrain (Table 4, Part 2*a*).

The other six flies transmitted trypanosome substrains which were related to both the reference and the ingested substrains. In these instances, the variant antigen was only partly replaced by the basic strain antigen during cyclical development, since antisera to the cyclically transmitted substrains had similar titres of agglutinins to the reference, ingested and transmitted substrains (Table 4, Part 2*b*). Absorption tests confirmed that these six tsetse flies transmitted trypanosomes which possessed a mixture of the basic strain antigen and those of the ingested serological variants.

The antigenic stability of the trypanosome strain in infective tsetse flies

The following experiment showed that the basic strain antigen responsible for the antigenic similarity of cyclically transmitted trypanosome substrains persisted in tsetse flies from the time they became infective until they died. Cyclically transmitted substrains were isolated from two rats infected by different tsetse flies and stored at −80°. Seventeen days later, just before they died, the flies infected two more rats from which a second pair of cyclically transmitted substrains was isolated. Sixth-day agglutinating antisera to the pairs of substrains were prepared in rabbits and each antiserum was tested for agglutinins to the homologous substrain and to the substrain from the other rat infected by the same fly. The pairs of substrains were closely related because, in both instances, an antiserum which had a high titre of agglutinins to the substrain isolated from the first rat had a similar titre to the substrain from the second rat (Table 5).

Table 5. *Titres of agglutinins in antisera to substrains of Trypanosoma brucei isolated from pairs of animals infected by the same tsetse fly*

Tsetse fly which transmitted the substrain	Substrain used as antigen	Titres of agglutinins in antisera to substrain			
		Rat 1	Rat 3	Rat 5	Rat 14
A	Rat 1	2560	320	na	na
	Rat 3	2560	320	na	na
F	Rat 5	na	na	1280	1230
	Rat 14	na	na	640	1230

na = not applicable.

The predominant character of the basic strain antigen

On several occasions during the preceding experiments, trypanosomes with the basic strain antigen developed at an early stage of infection when animals were infected with serological variants of the strain transmitted by syringe. Antigens with similar properties which occurred in clones of another strain of *Trypanosoma brucei* have previously been described as predominant antigens (Gray, 1965). The following experiment demonstrated the predominant nature of the basic strain antigen of the strain used in the present experiments. Three rabbits were infected by syringe with variants RFG, G 32 K and G 33 Q, which had been isolated in an earlier experiment at late stages of infection from Rabbit F, Goat 32 and Goat 33, respectively. Sera were collected from the rabbits before infection and afterwards at short intervals for 2–3 weeks. Sera from the rabbit infected with variant RFG were tested for agglutinins to substrain RFA with the basic strain antigen, and to the variants RFD, RFE, RFF and RFG, isolated originally from Rabbit F (see Fig. 1); similarly, sera from the rabbits infected with variants G 32 K and G 33 Q were tested for agglutinins to substrains with the basic strain antigen and variant antigens isolated from Goat 32 and Goat 33. The first antibodies produced by each of the rabbits agglutinated the variants with which they were infected. The next antibodies produced by the rabbits agglutinated substrains with the basic strain antigen, that is, substrains RFA, G 32 A and G 33 A. In several instances antibodies

to other antigens of the strain were produced later in the infections, but, in other cases, known strain antigens did not develop during the experiment (Fig. 4). Since the basic strain antigen of the cyclically transmitted trypanosome substrains developed before other known antigens in animals infected by syringe with serological variants, it may be described as a predominant antigen of the strain.

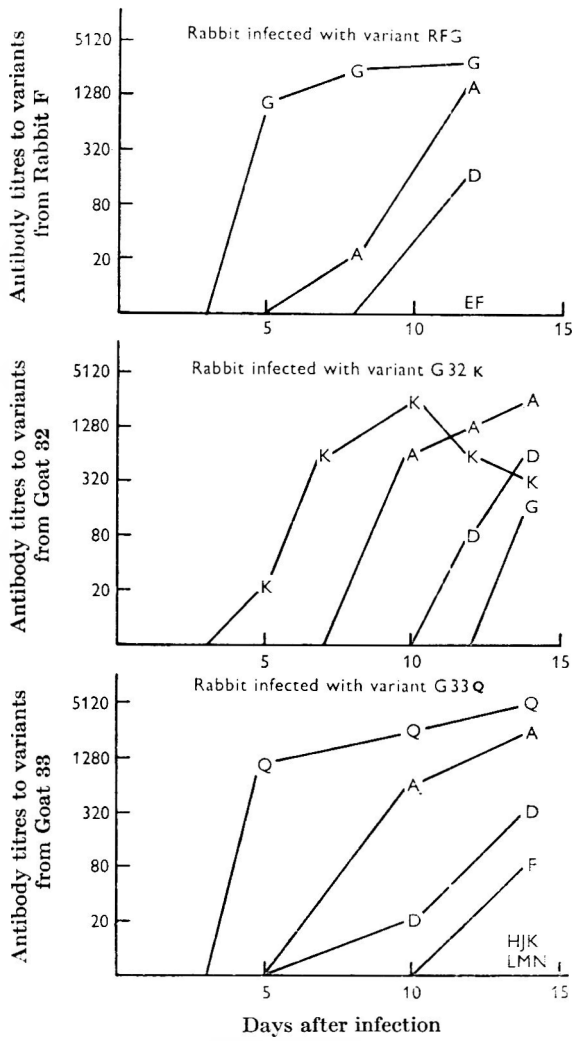

Fig. 4. The predominant character of the basic antigen of a strain of *Trypanosoma brucei*; agglutinin production by rabbits infected by syringe with serological variants, showing that trypanosomes with the basic strain antigen developed at an early stage of infection. A–A, antibody to the basic strain antigen; G–G, K–K, Q–Q, D–D, etc., antibody to variant antigens.

DISCUSSION

Many features of antigenic variation in *Trypanosoma brucei* in animals infected by tsetse flies are very similar to those in animals infected with rodent-adapted strains of trypanosomes transmitted by syringe. In addition to their capacity for

antigenic variation, the trypanosomes of a given strain seem to have an innate tendency to produce one particular antigen which has been provisionally called the basic strain antigen. While investigating the antigenic similarity of cyclically transmitted substrains of a strain, first described by Broom & Brown (1940) and amply confirmed in the present work, it was found that when trypanosomes with variant antigens were ingested by tsetse flies they were replaced during cyclical development by trypanosomes with the basic antigen. In some instances, ingested variant antigens were completely displaced by the basic strain antigen, but in most cases the change was incomplete, so that the tsetse flies transmitted substrains with a mixture of the ingested variant and the basic strain antigen. The possibility that the degree of reversion of the serological variants to the basic antigenic type of the strain was influenced by the ages of the infections in the flies was unfortunately not investigated, but it may be a factor of considerable importance. The period required for cyclical development of a strain of trypanosomes can vary considerably in different species of tsetse flies and under different experimental conditions. For example, the period required for complete cyclical development of the strain used in the present experiments was about 21 days in *Glossina morsitans*, while in *G. palpalis* it was at least 29 days. This problem should be considered in future work.

The innate tendency for trypanosomes of the strain to form a characteristic basic strain antigen was also apparent when serological variants were introduced into non-immune hosts by syringe; trypanosomes with the basic strain antigen appeared in the blood of infected animals as soon as antibody to the injected variant was produced. The concept of reversibility of antigenic variation in trypanosomes is not new; earlier workers have noted that variants of a strain sometimes revert to a 'parent' strain type during repeated sub-passage in mice and rats, that is, under conditions aimed at preserving variant characteristics by minimizing the effect of the immune response of the host (see Lourie & O'Connor, 1937). In this context, Inoki and his co-workers recently determined the proportions of different variants of a strain of *Trypanosoma gambiense* which developed in mice and found that a high proportion of those trypanosomes which appeared in the early stages of infections induced with different stocks of the strain were of one antigenic type (Osaki, 1959; Inoki, 1960; Inoki, Nakabayashi, Fukukita & Osaki, 1960).

The reversion of serological variants of a strain to substrains with the basic strain antigen during development in the tsetse fly may be simpler to explain than the host–parasite relationships by which strain characteristics such as morphology, pathogenicity and drug sensitivity are determined during cyclical transmission. Since some tsetse flies transmit trypanosome substrains with a mixture of the ingested variant and the basic strain antigen, it is unlikely that the basic strain antigen develops in association with any of the familiar morphological changes which occur when trypanosomes develop in tsetse flies (Wenyon, 1926), or even with fine ultrastructural changes of the type observed recently by electron microscopy (Vickerman, 1962). It seems more reasonable to suppose that variant antigens are gradually displaced by the basic strain antigen in the absence of host resistance. Although antibodies to some strain antigens are probably ingested by tsetse flies when they take blood meals from animals with chronic infections, and may suppress the development of antigens during the early stages of cyclical development, it is improbable that such antibodies exert any influence for as long as a week, since Weitz & Buxton (1953) and Cunningham, Harley, Southon & Lumsden (1962) have shown that blood proteins retain some serological specificity for about 4 days in the

gut of tsetse flies but are then rapidly denatured. Thus, the environment in which trypanosomes multiply in the tsetse fly is almost certainly free from antibody for at least 2 of the 3 weeks required for cyclical development and may favour the innate tendency of the strain to revert to the basic strain type.

Earlier experiments on antigenic variation have revealed little evidence of organization in the sequence in which the antigens of a strain develop. Experiments on this aspect of the problem have usually been limited to studies of the relationships of the first variants arising from a single stock of a strain in different hosts. Some workers believe that such first-relapse strains of one strain differ greatly (see Lourie & O'Connor, 1937), but others have found that they belong to a limited number of distinct antigenic types (Leupold, 1928; Osaki, 1959; Inoki, 1960). Few workers have followed the complete course of antigenic variation in experimental animals or compared the sequences in which the antigens of a strain develop in untreated infections in different hosts. Ritz (1916) showed that similar antigens developed in different rabbits infected with a clone of *Trypanosoma brucei* and Gray (1962), comparing the courses of antigenic variation in rabbits infected with a strain of *T. brucei*, found that the strain antigens developed in a well-defined pattern and that it was possible to predict, within limits, the order in which the antigens would develop from a given stock of the strain. The latter finding indicated that antigenic variation in trypanosomes was not such a random process as earlier work had suggested; this view is supported by results obtained in the present work. When several animals were infected with *T. brucei* by different tsetse flies, similar antigens developed in an identical sequence in the early stages of each infection; at later stages of infections, however, the order in which the antigens were produced varied, but many antigens developing in each infection were identical. In considering the nature of antigenic variation in trypanosomes, its ready reversibility and a degree of organization in the development of the antigens of strains seem to be more in keeping with a process of adaptation in the organisms than with one of mutation and selection of viable mutants, even though Watkins (1964) has produced evidence which seems to favour the latter hypothesis.

Although there is still some doubt about the relative importance of cyclical and mechanical transmission in the spread of African trypanosomiasis in certain areas (Buxton, 1955; Weitz, 1961), it seems that the mode of transmission of a strain is unlikely to be an important factor in controlling its antigenic constitution, since the basic strain antigen and other predominant antigens develop in new hosts infected in either way. It must be emphasized that this view is based on the results of experiments with a strain transferred from one host to another after maximum periods of infection of 30 days; greater or even permanent antigenic changes might occur in strains in more chronic infections and their antigenic characters might not be controlled by the means described. It would be interesting to see whether the basic strain antigen of a strain is sufficiently distinctive to enable it to be used to trace strains in epidemiological studies of trypanosomiasis.

The implications of these results in relation to the serological classification of trypanosome strains may be mentioned. The antigenic similarity of cyclically transmitted substrains of a strain suggests a basis on which strains of 'brucei' subgroup trypanosomes may be typed. In support of this, Broom & Brown (1940) and Gray (1963) showed that cyclically transmitted substrains of strains of *Trypanosoma brucei* from different localities were antigenically distinct but there are at

least three major difficulties to overcome before such a classification can succeed. Firstly, many strains of 'brucei' subgroup trypanosomes are very difficult to transmit cyclically in the laboratory and the basic strain antigen of a strain can only be identified initially in trypanosomes isolated from an infective tsetse fly; secondly, cyclically transmitted substrains of a strain may possess variant antigens as well as the basic strain antigen and the degree of cross-reaction between variant antigens of different strains is unknown; thirdly, newly isolated strains of 'brucei' subgroup trypanosomes sometimes cause only a low-grade parasitaemia in mice and could not be classified because of difficulties in obtaining enough trypanosomes for agglutination tests.

If further work shows that these difficulties cannot be overcome, it may still be possible to classify trypanosome strains on the basis of their predominant antigens. This method would avoid problems entailed in cyclical transmission and perhaps also the obstacle presented by less virulent strains, because antisera from hosts infected with such strains could be tested for antibody to a comprehensive range of trypanosomes having the basic and predominant antigens of more pathogenic strains. Either method alone, or a combination of both, would be an improvement on current methods of determining antigenic relationships of trypanosomal strains which amount to comparisons of the antigens of single populations of each strain chosen at random from the variety of antigenic forms in which any strain may appear (e.g. Soltys, 1957; Desowitz, 1961; Cunningham & Vickerman, 1962; Pautrizel, Duret, Tribouley & Ripert, 1962).

The detection of variant antigens, as well as a basic strain antigen, in cyclically transmitted substrains of a strain casts some doubt on the advisability of attempting to immunize animals against infection by means of trypanosomes possessing the basic strain antigen as suggested by Brown (1963). Immunization might be based more suitably on the bound or somatic antigens of trypanosomes, since such antigens are frequently common to several strains and species, whereas variable antigens have a very narrow range of immunological specificity (Gray, 1961; Weitz, 1962, 1963; Seed, 1963).

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Effects of Meso-inositol Deficiency on Some Important Biological and Chemical Characteristics of Yeast

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SUMMARY

Deficiency of meso-inositol (i-inositol, myo-inositol) caused decreased viability of *Saccharomyces carlsbergensis* ATCC 9080 and altered the internal morphology of the cells as seen in preparations stained with Nile Blue A, especially the nuclear membrane. Metabolic defects resulted which led to lipid accumulation in the organism and to increased concentrations of acetoin, acetaldehyde and glycerol in the culture medium. Hypotheses about the metabolic role of inositol are examined.

INTRODUCTION

Although meso-inositol (myo-inositol, i-inositol; in the sequel, inositol) is an essential nutrient factor for certain micro-organisms and some higher animals (mouse, rat, guinea pig, hamster, chick; Diem, 1962) and for human cells in tissue culture (Eagle, Oyama, Levy & Freeman, 1957) it has not been associated with a metabolic function which completely explains its role. Since Eastcott (1928) first reported that inositol was required for the normal growth of a strain of yeast, several investigators have used various inositol-requiring yeast species to study inositol metabolism. Schopfer, Posternak & Wustenfeld (1962) studied the effects of inositol deficiency and of administration of possible related anti-metabolites, with *Schizosaccharomyces pombe*, and observed marked changes in cellular morphology. They concluded that inositol functions as a regulator in cytogenesis and morphogenesis. Dawson, White & Freinkel (1962) separated inositol monophosphate, inositol-containing lipids and an unidentified neutral derivative of inositol from *Kloeckera brevis*, an inositol-requiring yeast. Challinor & Daniels (1955) and Challinor, Power & Tonge (1964) reported that an inositol-deficient strain of *Saccharomyces cerevisiae* accumulated acetone-soluble lipids. *S. carlsbergensis* grown in a deficiency of inositol has been shown to grow in aggregates, as contrasted with the normal organisms, which grow singly or with a single bud (Smith, 1951). Ghosh, Charalampous, Sison & Borer (1960) suggested that aggregation was due to a defective mechanism for synthesizing cell-wall material, since the walls of inositol-deficient yeasts were found to contain greater amounts of glucans than the walls of inositol-supplemented yeasts. In contrast with the work of Ridgway & Douglas (1958*b*), who utilized a different strain of *S. carlsbergensis*, Ghosh *et al.* (1960) reported that inositol-deficient yeasts retained their viability, exhibited a normal endogenous respiratory rate and were able to oxidize glucose, ethanol and acetate at normal rates. Ridgway & Douglas (1958*b*) observed unbalanced growth

of inositol-deficient yeasts, leading to death and accompanied by loss of cytochromes and nucleotide coenzymes. They suggested that the chief biochemical lesion involved the structure of a cytoplasmic granule, probably the mitochondrion (Ridgway & Douglas, 1958*a*, *b*). Since the above information has been obtained from observations on several different yeast species grown under differing conditions it seemed desirable to utilize one species in order to study the effects of inositol deficiency in detail. The work reported here is part of a study directed toward elucidating the biochemical functions of inositol in the inositol-requiring yeast strain, *Saccharomyces carlsbergensis* ATCC 9080. This organism is easy to grow on defined medium, shows dramatic inositol deficiency symptoms, has been well studied previously and has been used for the microbiological assay of inositol (Smith, 1951; McKibbin, 1959). The present report presents further data about the effects of inositol deficiency on the viability, growth rate, morphology, chemical composition and metabolic products of *S. carlsbergensis* ATCC 9080.

METHODS

Organism. Stock cultures of *Saccharomyces carlsbergensis* strain 4228 (ATCC no. 9080) were grown at 30° for 48 hr on Difco malt agar slopes and stored at refrigerator temperatures for 4–6 weeks. Samples (1 ml.) of a washed suspension of freshly grown *S. carlsbergensis* (Klett–Summerson colorimeter reading of 80–120 versus water blank; with no. 66 filter) were used to inoculate Erlenmeyer flasks (1 l.) containing 400 ml. of the inositol-assay medium of McKibbin (1959) modified by the omission of the casein hydrolysate and choline. Control flasks contained the above medium supplemented with inositol 5 mg./flask. Flasks were incubated at 28° with shaking (New Brunswick Gyrotory Shaker) at about 145 oscillations/min. Samples of culture medium were periodically removed aseptically and used for turbidity readings, microscopic examination and chemical analyses.

Determination of growth rate and cellular viability. The growth rate of the organism was followed turbidimetrically by using a Klett–Summerson photoelectric colorimeter with filter no. 66 and by direct microscopic count with a haemocytometer. The numbers of living and dead organisms were obtained by the methylene-blue vital staining technique of Fink & Kuhles (1933), in which dead organisms stain blue while living organisms remain colourless. Viable cell counts were also made by plating suitable dilutions on malt agar (Difco).

Microscopic examination after staining with Nile Blue A. Normal and inositol-deficient organisms were stained with alkaline Nile Blue A by the following modification of the method of Gancevici, Stoian & Keller (1962). A drop of yeast suspension, previously washed with sterile saline, was placed on a microscope slide, mixed with 1 drop of glycine buffer (0·1 M; pH 10) and 1 drop of Nile Blue A solution (0·02 %), and then examined under a coverslip at about × 1000 magnification. This technique clearly showed orange lipid inclusions, blue-green cytoplasm and a light purple nuclear compartment with 'dancing bodies'.

Lipid content of yeast organisms. Lipids were extracted from lyophilized yeast by the following modification of the method of Pedersen (1962). Samples of lyophilized yeast (about 1 g.) were mixed to a paste with chloroform + methanol (1 + 1, by vol.) and ground with small glass beads in a mortar and pestle for 10 min.

More chloroform + methanol mixture (10 ml.) was added, mixed, and the slurry filtered through a sintered-glass funnel (medium porosity). The extracted yeast was replaced in the mortar and the process repeated four times. Chloroform was added to adjust the extract to a composition of chloroform + methanol 2 + 1 by vol. before washing with $MgCl_2$ (0·0015 M) by the procedure of Folch, Lees & Sloane-Stanley (1957). The washed lipid extract was evaporated to dryness and held in a desiccator to constant weight.

Total lipid was separated into phospholipid and non-phospholipid fractions on silicic acid as described by Hirsch (1963). These lipid fractions were then evaporated to dryness and brought to constant weight in a desiccator.

Composition of growth medium. Samples of deficient and inositol-supplemented culture fluids were removed from the corresponding yeast cultures periodically and were centrifuged to deposit the organisms. The supernatant broth fluids were measured by standard methods for pH value and for the concentrations of glycerol (Lambert & Neish, 1956), acetoin (Westerfeld, 1945), acetaldehyde (method of Desnuelle & Naudet, described by Neish, 1952), and glucose (by glucose oxidase method; Keston, 1956). The partial pressures of oxygen and carbon dioxide (pO_2 and pCO_2) in Millipore-filtered liquid samples were determined using a blood gas analyser (Model 113, Instrumentation Laboratory, Inc., Boston, Mass., U.S.A.).

RESULTS

Loss of cellular viability

In our growth experiments, as in those of Smith (1951) and of Ghosh *et al.* (1960), it was observed that inositol-deficient organisms tended to grow in large aggregates. A vital staining method which would distinguish between living and dead organisms in a clump should therefore permit calculation of % viability and might provide information on the distribution of viable organisms within and among aggregates. For this purpose a vital staining technique based upon the staining of dead organisms by methylene blue was used to obtain the data of Table 1. Inositol deficiency apparently caused a distinct decrease in the viability of deficient organisms and the effect was annulled by the addition of inositol. Living and dead organisms were found to be distributed within the same aggregates. The number of dead organisms/ml. did not decrease after addition of inositol. The increase in % of viable organisms was therefore due to the production of many new viable organisms in the inositol-supplemented medium. Since methylene-blue staining has been reported to be a reliable indicator of viability only when the absolute viability of the culture is high (*European Brewery Convention* 1962) the viability of inositol-deficient and -supplemented cultures was also determined by plating suitable dilutions in malt agar. The results obtained were compared with those expected on the basis of the number of growth units/ml. observed by direct microscopic count. Cell aggregates and single cells each counted as one growth unit, since they would each lead to the formation of a single colony on a plate. These results (Table 2) also showed cellular death as a consequence of inositol deficiency.

Table 1. *The influence of inositol deficiency in the growth medium for Saccharomyces carlsbergensis* ATCC *9080 on the viability and on dissolved oxygen and carbon dioxide in the culture*

Samples were removed aseptically from the appropriate cultures at the times shown. Appropriate dilutions were prepared and viability was measured by the methylene-blue vital staining method of Fink & Kuhles (1933). The partial pressures of oxygen and carbon dioxide in the culture fluid were measured using a blood gas analyser, as described in the text. After 71·5 hr of incubation inositol (5 mg.) in 10 ml. sterile H_2O was added to the inositol-deficient cultures and a blank consisting of sterile H_2O (10 ml.) was added to the other cultures.

Time of incubation (hr)	Inositol + Viability (%)	Inositol − Viability (%)	Inositol + pO_2 (mm. Hg)	Inositol − pO_2 (mm. Hg)	Inositol + pCO_2 (mm. Hg)	Inositol − pCO_2 (mm. Hg)
0	—	—	158	150	11	< 10
24	—	—	80	128	> 100	58
50	97	46	—	124	> 10	35
71	94	10	142	158	15	15
Inositol added						
74	—	23	—	—	—	—
96	97	63	—	—	—	—
133	98	78	—	—	—	—

Table 2. *The influence of inositol deficiency in the growth medium for Saccharomyces carlsbergensis* ATCC *9080 on the viability of the organism, as determined by plate counting on malt agar*

Samples were removed from inositol-deficient and -supplemented cultures at the times shown, diluted appropriately for direct microscopic counts (in a haemocytometer) of total cell count, methylene-blue viable count, and for count of growth units/ml. Samples were also removed, aseptically, diluted serially with sterile water, and plated into malt agar. Colonies were counted after 48 hr of growth.

Time of incubation (hr)	Inositol + Direct microscopic count (cells/ml. $10^{-6}\times$)	Inositol − Direct microscopic count (cells/ml. $10^{-6}\times$)	Inositol + Direct microscopic count (growth units/ml.) ($10^{-6}\times$)	Inositol − Direct microscopic count (growth units/ml.) ($10^{-6}\times$)	Inositol + Plate count (colonies/ml.) ($10^{-6}\times$)	Inositol − Plate count (colonies/ml.) ($10^{-6}\times$)	Inositol + Viability of growth units (%)	Inositol − Viability of growth units (%)	Inositol + Viability by methylene-blue vital stain (%)	Inositol − Viability by methylene-blue vital stain (%)
45	175	32	155	5·0	156	3·4	100	68	99	67
68	167	46	136	6·7	132	3·2	97	48	99	55
93	171	53	143	7·8	130	1·7	91	22	99	48

Decrease in growth rate

For short periods of growth (about 20 hr) a proportionality has previously been shown to exist between the concentrations of inositol (from 0 to 1 μg./ml.) and the rate of growth of *Saccharomyces carlsbergensis* ATCC 9080, and this property has led to its use as an inositol assay organism (Smith, 1951; McKibbin, 1959). With longer periods of incubation Smith (1951) reported that the inositol-deficient organisms grew to about the same crops as did inositol-supplemented organisms, presumably

by slowly synthesizing some inositol. Our results of long period incubation (Fig. 1) show that inositol-deficient organisms eventually overtook normal organisms in total population as determined by turbidity and total counts. When cultures of inositol-deficient organisms were supplemented with inositol the growth rate increased rapidly (see Fig. 2) as the cells recovered from inositol deficiency.

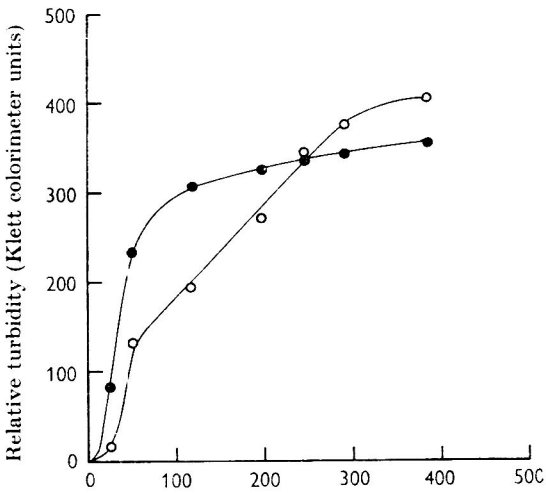

Fig. 1

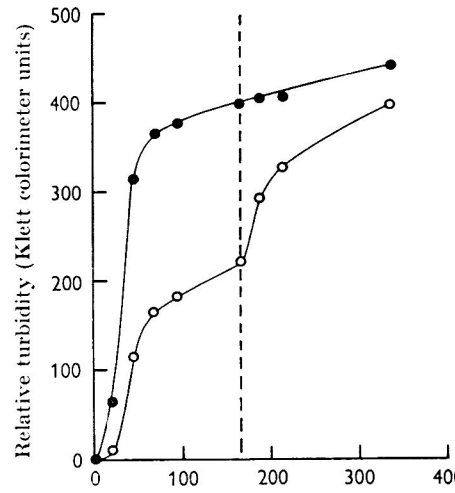

Fig. 2

Fig. 1. Growth of *Saccharomyces carlsbergensis* ATCC 9080 on media with and without inositol supplementation. ●, Growth with added-inositol; ○, growth without added inositol.

Fig. 2. The effect of adding inositol to *Saccharomyces carlsbergensis* ATCC 9080 growing in inositol-deficient medium. ●, Turbidity of cultures grown on medium with inositol supplementation (5 mg./400 ml.); ○, turbidity of cultures grown on medium to which inositol was added at the time shown by the vertical line (167 hr).

Changes in morphology

The earliest visible alteration in the morphology of inositol-deficient *Saccharomyces carlsbergensis* ATCC 9080 was the formation of cellular aggregates. This phenomenon of aggregation was most pronounced early in growth. With very long periods of incubation (350–400 hr) the inositol-deficient cultures contained large numbers of single organisms or small aggregates, as well as some large aggregates. This disaggregation may reflect a spontaneous recovery of the organisms upon long incubation, since Smith (1951) reported that inositol-deficient organisms were capable of some slow synthesis of inositol.

After incubation for about 48 hr, microscopic examination of preparations stained with Nile Blue A showed some alteration of the internal structure. Following Lindegren's terminology (see Winge & Roberts, 1958) the Nile Blue A-stained normal organisms after incubation for 75 hr showed a structure containing a large, light-purple nucleus, surrounded by small orange-staining globules (possibly mitochondria) in blue-staining cytoplasm. The nucleus or nuclear vacuole was not apparent in inositol-deficient organisms, which contained intracellular granules and orange lipid globules in blue cytoplasm. After somewhat longer

periods of incubation the cytoplasm of inositol-deficient organisms tended to lose its ability to retain blue and the orange lipid globules increased in size and fused to form large lipid droplets, similar to the lipid globules reported in *Saccharomyces cerevisiae* by Challinor & Daniels (1955). Some inositol-deficient *S. carlsbergensis* ATCC 9080 organisms appeared to contain one or two spherical dark-purple inclusions.

After addition of inositol to the deficient growth medium some *Saccharomyces carlsbergensis* ATCC 9080 organisms continued to contain colourless cytoplasm and orange lipid globules and showed no signs of recovery. These were presumably the non-viable organisms whose presence was detected by the methylene-blue vital staining technique. Other organisms in the aggregates recovered their ability to stain blue, developed light-purple nuclei and began to bud. In some cases purple material was seen to connect an adjacent organism and a bud. Finally, newly formed buds apparently separated from the aggregates to form normal organisms, as described by Ghosh *et al.* (1960). After recovery for 24 hr most of the viable organisms appeared as single or budding organisms, but there were still some aggregates present which contained both viable and dead organisms.

Inositol-deficient cultures tended to disaggregate after long periods (350–400 hr incubation). The resulting single organisms or small aggregates appeared to contain nuclei and blue cytoplasm and thus appeared to be more like normal organisms in their internal morphology than inositol-deficient organisms.

Changes in composition of cells and growth medium

The lipid material of normal and inositol-deficient cells was extracted and weighed (Table 3). Inositol-deficient organisms contained more lipid than did normal organisms. Fractionation on silicic acid indicated that the excess lipid from inositol-deficient organisms was mostly non-phospholipid.

Table 3. *Lipid content of normal and inositol-deficient Saccharomyces carlsbergensis ATCC 9080*

Lipids were extracted from lyophilized cells using chloroform + methanol (1 + 1, by vol.), as described in the text. Total lipid was separated into phospholipid and non-phospholipid fractions on silicic acid as described by Hirsch (1963).

Time of incubation (hr)	Inositol +	Inositol −	Inositol +	Inositol −
	Lipid (% of dry-wt.)		Non-phospholipid (mg.) / Phospholipid (mg.)	
70	8·7	13·4	1·2	4·5
112	7·8	13·9	·	·
599	9·8	13·1	5·6	6·5

Analysis of the growth medium (Table 4) showed that inositol-supplemented organisms decreased the pH value of the culture more rapidly than did inositol-deficient organisms; but the final pH values were similar. During their period of rapid growth the normal organisms decreased the oxygen tension and increased the CO_2 tension of the culture more dramatically than did the inositol-deficient cultures. Different products accumulated in the media during growth of normal and deficient organisms. We reported (Lewin & Smith, 1964) the accumulation of acetoin by inositol-deficient cultures. Acetaldehyde, a precursor of acetoin in

yeast, also accumulates (see Table 4). The results of experiments in which acetaldehyde was added to the growth medium at the start of incubation showed that inositol-deficient organisms were much more seriously inhibited by acetaldehyde than were normal organisms.

Table 4. *The effect of inositol deficiency in the growth medium for Saccharomyces carlsbergensis* ATCC *9080 on some metabolic products*

Samples of deficient and inositol-supplemented culture fluids were removed from the corresponding yeast cultures at the times shown, and were centrifuged to deposit the organisms. The supernatant fluids were assayed for pH value, acetoin and acetaldehyde as described in the text.

Time of incubation (hr)	Inositol ±	Inositol −	Inositol +	Inositol −	Inositol +	Inositol −
	pH value		Acetoin (μmole/ml.)		Acetaldehyde (μmole/ml.)	
0	4·8		0	0		0
24	4·4	4·7	0	0	2·1	1·3
44	4·0	4·5	2·1	1·0	4·0	7·3
72	3·8	4·3	4·7	5·7	3·9	10·6
96	3·9	4·2	5·8	6·2	1·7	8·0
192	3·9	3·9	4·9	13·3	1·7	8·4
214	3·9	3·8	4·1	13·7	3·9	7·6

Examination of the culture medium by paper chromatography indicated that there were several non-volatile compounds which reacted with alkaline silver nitrate reagent (Block, Durrum & Zweig, 1958) which were present in higher concentrations in inositol-deficient cultures than in normal cultures. The most prominent of these spots was glycerol. Subsequent analyses for glycerol (method of Lambert & Neish, 1950) showed that the inositol-deficient cultures accumulated up to four times as much glycerol as did the normal cultures (e.g. normal glycerol = 0·17 M; deficient = 0·07 M after incubation for 428 hr). Three other silver nitrate-reducing materials were present to a greater extent in the inositol-deficient than in the normal cultures. Similar chromatograms were examined under ultraviolet radiation and were sprayed with the ninhydrin, *p*-anisidine HCl and aniline acid phthalate reagents described in Block *et al.* (1958) without giving evidence for the presence of additional compounds present differentially in normal and deficient cultures.

DISCUSSION

Whether inositol deprivation causes loss of cellular viability has been contested in the literature. Ridgway & Douglas (1958*b*) reported that viable counts made by plating out suitable dilutions of *Saccharomyces carlsbergensis* on peptone yeast extract glucose agar indicated rapid loss of viability in inositol-deficient organisms. Ghosh *et al.* (1960), with *S. carlsbergensis* ATCC 9080, did not detect any evidence for cellular death caused by inositol deficiency. They noted that aggregation of inositol-deficient organisms complicated the interpretation of colony count data and therefore attempted to disrupt the aggregates by ultrasonic treatment before plating. This procedure, however, resulted in some disruption and death of organisms. On the basis of their data they stated that single organisms and aggregates

of inositol-deficient organisms maintained their ability to form colonies on agar plates. The data presented here indicate that inositol-deficient *S. carlsbergensis* ATCC 9080 suffered a definite loss in viability as measured by plating on to malt agar and by methylene-blue vital count. The difference between these conclusions and those of Ghosh *et al.* (1960) may be explained by noting that their viability determinations were made only on cultures grown for short times of incubation (up to 41 hr) when viability of inositol-deficient cultures might still be relatively high.

The data reported here confirmed the finding of Smith (1951) that the initial depression of the growth rate of inositol-deficient *Saccharomyces carlsbergensis* ATCC 9080 does not prevent the cultures from eventually reaching at least the same total population as that of inositol-supplemented organisms. Addition of inositol to the deficient organisms results in a rapid increase in the growth rate. The organisms newly formed upon recovery from inositol deficiency appear to separate from aggregates, as described by Ghosh *et al.* (1960) and appear to have normal internal morphology, as seen in preparations stained with Nile Blue A.

The internal structure of inositol-deficient *Saccharomyces carlsbergensis* ATCC 9080 appears to be deranged. Both the disappearance of the nuclear vacuole and the appearance of lipid globules in inositol-deficient organisms may be signs of damage to cellular membranes, since inositol phosphatides may play a role in their integrity.

The lipid material accumulated in inositol-deficient organisms was mostly nonphospholipid, in agreement with the results of Challinor & Daniels (1955), who found that acetone-soluble lipids were accumulated by inositol-deficient *Saccharomyces cerevisiae*. Since lipids have also been reported to accumulate in inositol deficient *Neurospora crassa* (Shatkin & Tatum, 1961) and since inositol has been reported to function as a lipotropic agent in higher animals (see discussion in Deuel, 1955), we shall later examine the lipids accumulated by inositol-deficient *S. carlsbergensis* ATCC 9080.

Since it appeared reasonable to assume that normal and inositol-deficient *Saccharomyces carlsbergensis* ATCC 9080 would differ in their rates of utilization of components of the growth medium and in the amount (and possibly in the nature) of the products excreted into the medium, an examination of the culture fluid might be expected to provide information about metabolic lesions in inositol deficiency; also, some of the visible signs of inositol deficiency might be caused by differences in the culture fluid medium which were secondary in nature. As reported previously (Lewin & Smith, 1964), glucose utilization by inositol-deficient *S. carlsbergensis* ATCC 9080 cultures was slower than that of normal ones, but eventually all of the glucose in the medium was utilized. During their rapid growth and glucose utilization normal organisms decreased the pH values of the culture more rapidly than did inositol-deficient cells, but the final pH values were similar, indicating that about equal amounts of acid were produced. Larger amounts of acetoin, acetaldehyde and glycerol were found in cultures of inositol-deficient organisms than in normal organisms. From the products accumulated it would seem that inositol-deficient organisms were capable of forming hydroxyethylthiamine pyrophosphate (HETPP), which is a precursor of acetaldehyde, acetoin and acetyl-CoA. If the citric acid cycle were not functioning normally in inositol-deficient organisms one might expect more acetyl-CoA to be used for lipid synthesis

and HETPP to be used to a greater extent than used for the formation of acetaldehyde and acetoin. It has long been known that acetaldehyde production in yeast fermentation may be correlated with the accumulation of glycerol therein (Nord, 1958). Abnormal function of the citric acid cycle might be expected to occur if the mitochondria were abnormal. Vignais, Vignais & Lehninger (1964) showed that phosphatidylinositol is a specific compound essential for the contraction of aged mitochondria; Ridgway & Douglas (1958*a*, *b*) suggested that inositol-deficient yeast have abnormal mitochondria.

A deficiency of inositol in the culture medium during aerobic incubation resulted in definite quantitative alterations in accumulation of metabolic products by *Saccharomyces carlsbergensis* ATCC 9080. Since preliminary experiments have shown differences in morphology of organisms and composition of the culture media in anaerobically and aerobically grown inositol-deficient organisms, work is in progress designed to examine the results of inositol deficiency under anaerobic conditions. The inositol-deficiency symptoms (and particularly the accumulation of excess acetoin, acetaldehyde and glycerol) reported here may reflect the lack of inositol or a derivative of it which functions as a cofactor in some enzymic reaction(s). It is also possible that this accumulation of metabolic products may reflect the disorganization of intracellular structure which was seen by microscopic examination of the inositol-deficient organism.

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An Alternative Method of Isolating the Membrane of a Halophilic Bacterium

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SUMMARY

The membrane of *Halobacterium halobium* was isolated after rupturing the organisms osmotically in 0·02 M-$MgCl_2$. Selected properties of this membrane were compared with those of membranes isolated from organisms which had been ruptured mechanically in 5 M-NaCl. The two types of preparation were indistinguishable from one another in the electron microscope, but some differences were noted between the present series of preparations and those described earlier. These differences, of which the occasional retention of the characteristic surface pattern of this organism was notable, were attributed to a slight modification of fixation conditions used in the present work. There were no major chemical differences between the two types of preparation but some minor differences in H^+ titration and carbohydrate analyses possibly reflected slight structural differences between the two types of preparation. The osmotic method described here is much quicker than the mechanical one and, unlike the mechanical one, the time involved is largely independent of the quantity of organisms being used.

INTRODUCTION

The properties of a complex subcellular component isolated from a living organism are presumably dependent to some extent on the isolation procedure used. Lipoprotein membranes are not likely to be an exception to this generalization (see for example Ponder, 1961) and it is a reasonable assumption that an isolated membrane differs in various ways from the functional structure in a viable organism. The opportunities for isolating a microbial membrane by more than one method, however, are limited. Membranes from Gram-positive bacteria and structurally related organisms are normally obtained by digesting the cell wall enzymically (with risk of changes in the membrane) followed by osmotic rupture of the protoplast. At the present time there is apparently no alternative to this. The two membranous components of the envelope of most Gram-negative bacteria are not easy to separate from each other but together they can be separated from the rest of the cell by ultrasonic treatment or by any of several fundamentally similar mechanical methods of rupturing the organism. The extremely halophilic bacterium *Halobacterium halobium* is bounded by a single lipoprotein membrane (Brown & Shorey, 1963) which hitherto has been isolated only by mechanical breakage of the organisms in the presence of high concentrations of NaCl followed by centrifugation in salt solutions. Because these organisms grow in high concentrations of NaCl they can be subjected, by dilution of their suspending medium, to a very large

osmotic shock. Such a simple dilution procedure cannot be used for isolating the membrane, however, because the membrane itself dissolves at decreased salt concentrations (Brown, 1964). Membrane dissolution is caused by electrostatically induced disaggregation of the structure and can be prevented by high concentrations of monovalent cations or relatively low concentrations of bivalent cations (Brown, 1964). Concentrations of bivalent cations which are sufficient to stabilize the membrane against electrostatic dissolution are insufficient to stabilize the organism against osmotic rupture. This fact has been used to provide an alternative method which is quicker than mechanical breakage of the organisms, and which can be used on a larger scale than the mechanical or ultrasonic techniques so far available to the authors. Slight differences in the properties of membranes isolated by osmotic lysis on the one hand, and mechanical disintegration on the other, are relevant to an ultimate understanding of membrane structure.

METHODS

Organism. Halobacterium halobium was grown for 3 days at 30° with rotary agitation. The growth medium contained Oxoid peptone (1%, w/v) in the salts solution described by Sehgal & Gibbons (1960).

Isolation of cell membrane. The culture was centrifuged at 2600 *g* for 20 min. The deposit was divided into two parts and each half treated separately as follows. (i) One half was suspended in 5 M-NaCl at 4° and portions of this suspension were shaken with glass beads in a Mickle tissue disintegrator for 30 min. in an ambient temperature of 4°, with intermittent cooling in an ice bath (Brown, 1961). For this purpose the organisms from 1·5 l. of culture were suspended in 80 ml. 5 M-NaCl, and 20 ml. portions were shaken at a time. The time involved in this disintegration step, or with any other batch-type mechanical device, is proportional to the volume of suspension being processed. After 30 min. the beads were removed on a coarse sintered-glass filter. The membrane suspension was centrifuged for 1 hr at 14,000–17,000 *g* and thereafter purified by a series of fast and slow depositions in a centrifuge (Brown, 1961). Throughout the whole procedure the membranes were suspended in cold 5 M-NaCl. (ii) The second half of the centrifuged deposit was suspended, with rapid mixing, in 0·02 M-$MgCl_2$ at 4°. For this purpose the organisms from 1·5 l. of culture were suspended in 80–100 ml. 0·02 M-$MgCl_2$. The organisms lysed virtually instantaneously and the suspension became unmanageably viscous with the release of deoxyribonucleic acid. The suspension was incubated at room temperature with sufficient deoxyribonuclease (DNase, about 2 mg. in 250 ml.) to decrease the viscosity effectively within 20 min. The time lapse between suspending the organisms in $MgCl_2$ solution and completing incubation with DNase was normally about 30 min. and was largely independent of the mass of organisms (or volume of bacterial suspension). The suspension was then centrifuged at 14,000–17,000 *g* for 1 hr and resuspended in cold 5 M-NaCl. Sometimes it was found at this stage that not all the bacteria were broken, in which case the suspension in 5 M-NaCl was centrifuged and the deposit again suspended in 0·02 M-$MgCl_2$ followed by incubation with DNase as before. After this second treatment virtually all the organisms had lysed. The extent of breakage on the first dilution was affected by the speed of mixing and by the volume of suspending solution. When the osmotically lysed bacteria were

eventually resuspended in cold 5 M-NaCl they were fractionated in a centrifuge under conditions identical with those used for the mechanically broken half of the preparation. In both cases depositions in a centrifuge were continued until the appearances of the supernatant fluid and of the membrane suspension (the latter in a phase-contrast microscope) were consistent with adequate freedom from cytoplasmic contamination. This was usually achieved in 3–4 cycles of fast and slow depositions.

Electron microscopy. The techniques used were described fully by Brown & Shorey (1963). Membrane preparations were fixed with buffered $KMnO_4$ followed by uranyl nitrate as described except that in the present work the membranes were suspended in 5 M-NaCl instead of 'half-strength salts' up to the time of fixation. Fixed membranes were washed three times in water, deposited from aqueous suspension on to carbon-coated nitrocellulose supporting film and shadowed with gold-palladium or else they were embedded and sectioned. For the latter purpose they were dehydrated conventionally with ethanol, passed once through xylene, embedded in Araldite and sectioned (Brown & Shorey, 1963). Both shadowed and sectioned specimens were examined in a Philips E.M. 200 electron microscope.

Chemical examination. Membrane suspensions were titrated at constant ionic strength in 4 M-NaCl with 0·02 N-NaOH and HCl. Details and implications of this technique, as applied to membranes, are discussed more fully elsewhere (Brown, 1965). In the present work, reference solutions of lipoprotein were not used since the aim of the titrations was merely to compare the two types of membrane preparation. After titration, membrane suspensions were dialysed exhaustively against distilled water at 4° and the resulting lipoprotein solutions (Brown, 1964) were freeze-dried and weighed. Protein (Lowry, Rosenbrough, Farr & Randall, 1951), total phosphorus following digestion with H_2SO_4 and HNO_3 (Fiske & Subbarow, 1925), amino sugar (Rondle & Morgan, 1955) and total hexose (Dische, 1955) were estimated in these preparations. The last two estimations were made on acid-hydrolysed (2 N-HCl for 2 hr at 100°) lipoprotein.

RESULTS AND DISCUSSION

Electron microscopy. Shadowed membranes looked similar after both methods of isolation (Pl. 1, figs. 1–3) and, with one important exception, the same as encountered previously by Brown & Shorey (1963). The exception was that a few individual membranes retained the surface pattern noted by Houwink (1956) on whole organisms of this bacterium and subsequently by Kushner *et al.* (1964) on unfixed membranes isolated from *Halobacterium cutirubrum*. This pattern was not encountered previously by Brown & Shorey (1963) in membranes suspended in 'half-strength salts' up to the time of fixation; it is likely that the use of 5 M-NaCl in the present work assisted in preserving the surface structure. The pattern was encountered more frequently in membranes isolated by mechanical breakage of the organisms than by osmotic lysis but not enough preparations were examined to allow any statistical evaluation of this statement.

When examined in thin section there was no apparent difference between membranes isolated by either method (Pl. 1, fig. 4; Pl. 2, figs. 1, 2) or, with an occasional exception, between the current series of isolates and those described by Brown &

Shorey (1963). The occasional exception was found in parts of membranes which resembled compound rather than unit membranes (Pl. 2, fig. 2). This kind of appearance was encountered previously by Brown & Shorey (1963) in sections of whole organisms of *Halobacterium halobium* but was not seen in isolated membranes. Like the surface pattern, the compound membrane appearance in isolated membranes was probably a result of the different ionic conditions during isolation. The compound appearance was encountered after both methods of isolation.

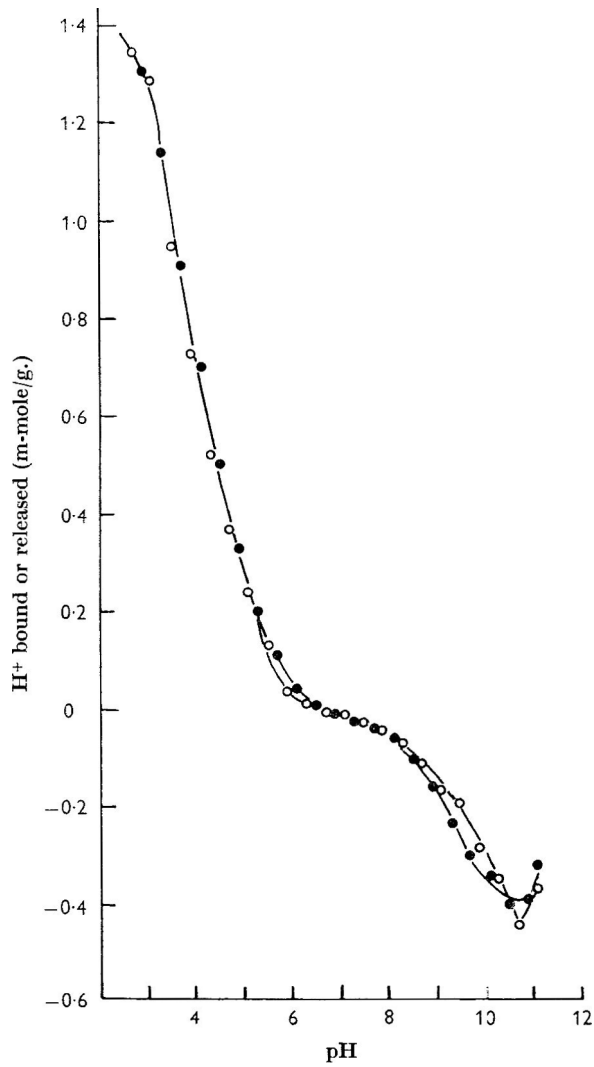

Fig. 1. Hydrogen-ion titrations of membranes (preparation 2) prepared by mechanical disintegration (○——○) and osmotic lysis of organisms in the presence of $MgCl_2$ (●——●).

Hydrogen-ion titrations

Titration curves of membranes isolated from the same culture by both methods are shown in Fig. 1. Many of the basic groups present on the lipoproteins of this organism do not titrate in the intact membrane and are presumed to be 'buried'

in the deeper parts of the structure; α-NH_2 groups become titratable when the membrane swells, even slightly, in an ion deficit (Brown, 1965). This method of analysis is thus quite a sensitive one for detecting some subtle changes in membrane structure. The numbers of groups titrating in three pairs of preparations are shown in Table 1; from this it is apparent that pH 6·0–8·2 was the only titration range in which there was a consistent difference between the two types of preparation, the osmotically lysed preparation having the higher titre. Groups titrating in this range include imidazole (of histidine), phosphate (pK_2 if phosphate mono-esters are present) and α-NH_2. The fact that pH 6·0–8·2 was the only range in which differences between the two types of preparation were consistently obtained is in accord with the previous finding (Brown, 1965) that the bonds responsible for 'burying' α-NH_2 groups in this membrane are weak and easily disrupted. For reasons apparent from a fuller treatment of this topic (Brown, 1965) it is thought that in the present series of preparations the differences in titre were caused by the exposure of groups which were previously 'buried' rather than the converse. It is possible that a higher concentration of $MgCl_2$ at the time of lysis would have prevented these slight differences in titre from arising but this was not investigated. In preliminary observations it was noted that an increase of $MgCl_2$ concentration to 0·025 M was accompanied by the rupture of noticeably fewer organisms.

Table 1. *Hydrogen-ion titrations*

Numbers of groups titrating in 4 M-NaCl on three preparations of membranes isolated by both methods.

pH range	Groups titrating (theoret. pK)	Preparation	H^+ bound or released (m-mole/g. membrane) Mechanical	Osmotic
2·8– 6·0	α-COOH (3·6)	1	1·21	1·36
	β-and γ-COOH (4·6)	2	1·27	1·30
	Imidazole (6·2)	3	1·24	1·19
6·0– 8·2	Imidazole (6·2)	1	0·07	0·15
	Phosphate (pK_2, 6·5)	2	0·09	0·12
	α-NH_2 (*c.* 7·5)	3	0·13	0·15
8·2–10·0	α-NH_2 (*c.* 7·5)	1	0·16	0·12
	Tyrosine-OH (9·5–9·8)	2	0·24	0·27
	ϵ-NH_2 (10·4)	3	0·20	0·18
10·0–11·0	Tyrosine-OH (9·5–9·8)	1	*	*
	ϵ-NH_2 (10·4)	2	*	*
		3	0·22	0·24

* Titration had an alkaline end-point below pH 11·0.

The reasons for selecting these ranges of pH in which to compare titres are discussed elsewhere (Brown, 1965).

Chemical analyses

Table 2 summarizes results of chemical analyses of four classes of substance frequently used to characterize biological membranes and related structures. In one preparation lipoprotein from mechanically isolated membrane contained more phosphorus and the same amount or slightly less protein than its osmotically isolated counterpart; in the other preparations the converse was true. Thus variations in the proportion of phosphorus and protein cannot be attributed to the methods used for rupturing the organisms. Consistent differences were obtained,

however, in the two sets of carbohydrate analyses; both amino sugar and total hexose were consistently higher in the lipoproteins isolated from mechanically ruptured organisms. It is not yet known whether these carbohydrates are part of polysaccharide present in small amounts in the membrane or of glycoprotein. Probably the amino sugar and hexose originated at least partly in different polymers, since different proportions of each were 'lost' on osmotic lysis. In two of three preparations the osmotically isolated lipoprotein contained about 40 % less hexosamine and about 30 % less hexose than did lipoprotein from mechanically ruptured organisms; the differences were less in the third preparation. For a component to differ by as much as 40 % between preparations without reflecting the difference in other analyses, it must either be present in very small amounts or, alternatively, it must contain the other components analysed, namely phosphorus and protein, in the same proportion as the whole membrane; such a compound would be a lipoglycoprotein. Variations in the proportions of the components which contained the carbohydrates were not reflected in the electron microscopy of the membranes, but were correlated with differences in H^+ titre in the range pH 6·0–8·2. It is not known whether there is any direct causal relationship between these two analytical differences but preparation 3, which had the smallest difference in carbohydrate analyses, also had the smallest difference in titre (pH 6·0–8·2) between the two types of preparation.

Table 2. *The content* (%) *of various substances in lipoprotein from membranes isolated by the two methods*

Preparation	Phosphorus	Protein	Amino sugar (as glucosamine)	Hexose (as glucose)
1. Mechanical	1·17*	76	1·03	3·28
Osmotic	0·68	79	0·60 (58 %)†	2·32 (71 %)
2. Mechanical	1·02	75	0·87	2·50
Osmotic	1·22	73	0·50 (58 %)	1·73 (69 %)
3. Mechanical	0·61	77	0·84	2·42
Osmotic	0·74	73	0·72 (86 %)	1·99 (82 %)

* Figures are the means of duplicate estimations which deviated from the mean by less than 5 % except in one case in which the deviation was 9 %.

† Bracketed numerals are analytical results for the osmotic preparation expressed as a percentage of the corresponding result for the mechanical preparation.

Conclusion

It is thus evident that membranes isolated from *Halobacterium halobium* by osmotic lysis in 0·02 M-$MgCl_2$ retained their morphological identity. In an electron microscope they could not be distinguished with certainty, if at all, from membranes isolated by mechanical breakage. There were no major chemical differences but some minor ones possibly reflected very slight structural differences between the two types of preparation. The importance of such slight differences will depend on the purpose for which the isolated membrane is required. The osmotic method is much quicker and more convenient than the mechanical method with which it was compared and is now being used routinely to obtain membrane lipoproteins for chemical and physico-chemical studies.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Membranes isolated by mechanical disintegration of the cells. The surface pattern of this organism has been preserved in one membrane (*A*) and partly in another (*B*). × 15,000.

Fig. 2. The membrane (*A*) from Fig. 1 shown at higher magnification. This pattern, which has been observed on whole cells and unfixed membranes deposited from suspension in salt solution, has not previously been obtained in fixed membranes. The identity of the larger lumps is not known but they appear to be caused by material occluded within the cell envelope. × 45,000.

Fig. 3. Membranes isolated by osmotic lysis in the presence of $MgCl_2$. The larger membrane has evidently collapsed lengthwise and shows the hole where the cell burst. Patterned membranes were occasionally seen in lytic preparations but no examples as good as Fig. 2 were obtained. × 45,000.

Fig. 4. Section of a membrane isolated from mechanically ruptured cells. × 100,000.

PLATE 2

Fig. 1. Section of a membrane isolated from osmotically ruptured cells. × 100,000.

Fig. 2. Section of membranes isolated from mechanically ruptured cells. Parts of these membranes resemble compound membranes. This appearance was also noted occasionally in membranes isolated by the osmotic method and has previously been reported (Brown & Shorey, 1963) in sections of whole organisms. × 100,000.

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The Isolation and morphology of Some New Bacteriophages Specific for Bacillus and Acetobacter species

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SUMMARY

The best natural habitat for bacteriophages is probably a semi-solid medium containing actively dividing host bacteria. Such conditions are provided for Bacillus and Acetobacter species in rotting grass and apples, respectively. The Bacillus phages found included one with a large head and a contractile tail, and also a so-called killer particle, which had a 350 Å head and a long contractile tail. This particle had the property of killing but not multiplying within a sensitive cell. A new morphological type of virulent Bacillus phage was also isolated; its head was oblong and the tail consisted of a short needle and a plate. The one Acetobacter phage found resembled coliphage T3 but was of particular interest because of the prominence of the head capsomeres and the three-pronged tail.

INTRODUCTION

Whether by accident or design, the emphasis in phage research has been placed on phages specific to *Escherichia coli* and other members of the Enterobacteriaceae. Recently, however, the obvious structural, taxonomic and physiological importance of studying a large number of phages for a wide range of host bacteria has led to their examination in the electron microscope by many workers. These results have been reviewed elsewhere (Bradley, 1965*a*), and it must now be asked whether it is of value to continue to study phages in the electron microscope on this basis. It seems logical to limit descriptions to new morphological types, or phages of particular chemical or physiological interest such as those specific to unusual host bacteria. It is also considered important to study those isolates from natural habitats on hosts not belonging to the Enterobacteriaceae. The genus *Bacillus* is particularly widespread, and is chosen because its bacteriophages have been somewhat neglected in spite of the fact that they are plentiful in natural habitats. The best natural habitat for a phage is where its host is actively dividing, preferably in a semi-solid medium, for micro-ecological reasons (Bradley, 1965*a*). Thus, while Bacillus phages are available in the soil, a better habitat would be decomposing vegetation, and this has proved to be the case, in the present work, with rotting lawn mowings.

The usual method for isolating phages consists of plating a suitable laboratory strain of host organism with a cleaned extract of habitat material. Better results can be obtained by isolating the bacteria from the habitat material and using them as potential phage hosts. In the present paper, the examination in this way of rotting lawn mowings and apples is described. In the apples, acid conditions provide

a microflora dominated by yeasts, Acetobacter, and perhaps Micrococcus species. The electron microscope shows that the phages isolated from both sources are of particular morphological and physiological interest; infectivity tests revealed that one of them was a killer particle (Seaman, Tarmy & Marmur, 1964).

METHODS

Plating media and methods. Standard media and procedures were used throughout as follows. The Bacillus species from grass were grown on nutrient broth agar consisting of (g./l.): beef extract, 2; yeast extract, 4; peptone, 10; NaCl, 10; agar, 20; pH 7·4. For the double agar layer method of Adams (1959), 1% (w/v) agar + 1% (w/v) peptone was used for the soft layer.

Malt extract agar was used for the initial growth of the Acetobacter species (g./l.): malt extract, 17; mycological peptone (Oxoid), 3; agar, 30; pH 5·4. Soft agar layers were made from the same medium but with only 15 g. agar/l. For phage propagation it was found that better results were obtained with nutrient broth (pH 7·4).

These media without agar were used for isolating bacteria from the two habitats.

Isolation of host bacteria. Samples of rotting grass or apple were shaken with 5–10 ml. of the appropriate liquid medium. A loopful of this mixture was streaked on the appropriate nutrient agar plate and incubated overnight at 30° or 37°. The colonies obtained were selected according to their morphology and further purified by streaking, before growth on slopes. The use of an acid medium with the rotten apple samples ensured that Acetobacter species and yeasts dominated. Occasionally the dominance of Bacillus species was ensured by heating the extract from the rotting grass at 80° for 20 min., leaving the Bacillus spores viable. The heated extract was then streaked on plates.

Isolation, purification, and growth of phages. The extract of habitat material obtained as described was centrifuged at 2000 *g* for 10 min. to remove bacteria; sometimes an extract was shaken with a little chloroform before centrifugation to help to decrease viable bacteria. (A residue of chloroform can inhibit bacterial growth in subsequent procedures.) A sample (0·3 ml.) of this extract was plated out by the double agar layer method (Adams, 1959) with each of the bacterial isolates obtained above. Phages formed plaques after incubation overnight. These plaques were picked with a platinum loop and suspended in about 1 ml. appropriate liquid medium. Purification was achieved by several cycles of plating and plaque picking.

In the case of the Acetobacter phage (AA-1), no plaques were seen when an extract of rotting apple was plated with host bacteria. To ensure that plaques were not missed because of bacterial overgrowth, the plate was extracted with liquid medium, the extract centrifuged to deposit bacteria, and then tested for activity against the original host by the spot test. A positive result indicated the presence of a phage; it was grown to high titre as described below.

High-titre phage preparations for electron microscopy were obtained by confluent lysis of host bacteria on 6 in. plates and extracting the phage with neutral 0·1 M-ammonium acetate. Then extracts were cleaned by alternate cycles of centrifugation at 2000 *g* for 15 min., and at 10,000–15,000 *g* for 4 hr (angle head). The final pellet was re-suspended in 0·1–0·2 ml. neutral 0·1 M-ammonium acetate per plate extracted.

A mixture of the dominant phages in a sample of habitat material was obtained by plating 1 ml. liquid medium extract by the double agar layer method. Extraction of the plate with 0·1 M-ammonium acetate followed by cycles of centrifugation as above produced a phage suspension suitable for electron microscopy.

Suspensions of killer particles for electron microscopy were prepared by extracting a double agar layer plate of host bacterium with about 3 ml. 0·1 M-ammonium acetate; this extract contained a high concentration of spontaneously released particles. The general appearance in the electron microscope suggested that about 10^8 particles were extracted from each 3 in. diameter plate.

The spot test for phage activity. This was done as previously described (Bradley, 1964) by placing a drop of phage suspension on a double agar layer plate of host bacteria and observing clearing or otherwise after incubation. The test was also used to distinguish killer particles from virulent phages as follows. Both killer particles and virulent phages give cleared areas with the spot test at high concentrations. However, when a series of dilutions is spotted on a plate, the cleared area will become uniformly more opaque as the dilution is increased. With a virulent phage the clear area will remain clear until with increasing dilution it will become broken into a series of discrete plaques. But a killer particle suspension giving a positive spot test will form no plaques when plated out in serial dilutions. The reason for this is obvious: a killer particle destroys one host cell, but a multiplying virulent phage many. The killer particle's 'plaque' will thus consist of the area occupied by one bacterium, and be invisible. If there are enough killer particles in a small area of plate, they will destroy all the bacteria, creating a clear area, and thus showing a positive spot test.

Table 1. *Numbers of phages and hosts*

Phage no.	Host bacterium species	Host strain no.
GA-1	*Bacillus subtilis*	G1R
GA-2	*B. subtilis*	G1R
GF-2	*B. subtilis*	G6
AA-1	*Acetobacter* sp.	A2

The release of killer particles or temperate phages from a bacterium can be detected by spotting a suspension of the suspected bacterium on a double layer plate of the supposedly sensitive host. After incubation, the surface colony will be surrounded by a clear border in the soft agar if it releases particles active against the bacterium under test. This was used to demonstrate the similarity of the killer particle GA-2 to a temperate phage.

Electron microscopy. The standard negative staining procedure (Brenner & Horne, 1959) with neutral 2% (w/v) potassium phosphotungstate was used throughout.

Identification and numbering of bacteria and phages. Members of the genus *Bacillus* were identified at species level and those of *Acetobacter* at the generic level by standard tests done at the Department of Agriculture, University of Edinburgh, and in the case of *Bacillus* spp. also by spore morphology (Bradley & Franklin, 1958). To avoid confusion arbitrary laboratory numbers are quoted as well (see Table 1). Arbitrary numbers were also given to phage isolates in the absence of any recognized system of nomenclature.

RESULTS

Bacillus subtilis phage GA-1

This new morphological type is shown in Pl. 1, figs. 1–6. It has an oblong head with a six-sided outline of length 570 Å and width about 400 Å. The short tail is a tube 60 Å wide and 350 Å long. Part of the way down there is a plate-like structure attached to the base of the head by fibrous material. The plate has several downward-pointing prongs (Pl. 1, figs. 4, 5); three are most often visible, but some particles (see the one at the bottom left in Pl. 1, fig. 1) appear to have four. The prongs are about 70 Å long and are attached to prominent subunits on the plate. The micrographs also illustrate some other points. In Pl. 1, fig. 2, the top particle has lost part of its contents; head capsomeres can just be seen, but not in a clear arrangement. Another particle has lost its tail and shows a flattening at the point of attachment of the tail. This is also shown particularly clearly in Pl. 1, fig. 2, the particle here being completely full; the micrograph also shows the fibrous material between the plate and the head. In Pl. 1, fig. 3, fibrous material is again visible together with the point of attachment between the tail and head, where there is a plug of protein, commonly found in all tailed phages. A comparison of the tails in the empty or partly empty virions in Pl. 1, figs. 1, 3, with the full one in Pl. 1, fig. 2, shows that the former are hollow and the latter solid. This feature has been observed particularly in phages with long non-contractile tails (Bradley & Kay, 1960). In Pl. 1, fig. 6, two particles are shown attached to a piece of debris; this suggests that adsorption takes place tail-first in the conventional manner.

Bacillus killer particle GA-2

An intact killer particle GA-2 is shown in Pl. 1, fig. 8; it consists of a small head with a massive tail. The head is 350 Å in diameter, but the tail is 2100 Å long and has the conventional appearance of a contractile type with a striated sheath and tail fibres. The number of tail fibres is difficult to ascertain; 4 or 5 is most likely. An empty head and short length of tail core are shown in Pl. 1, fig. 7. The core is in the form of a hollow tube about 90 Å in diameter and there is a lump of protein at the attachment to the head. The head membrane is continuous and does not show any obvious capsomeres. The particle in Pl. 2, fig. 9, has a partly empty head and shows a regular hexagonal outline. The tail capsomeres are clearer on the headless tail (Pl. 2, fig. 10) and they are undoubtedly tubular in form (end-on capsomeres arrowed) and are probably arranged in screw symmetry. Plate 2, fig. 11, shows a virion with a contracted tail, though the head has remained full. Here, the top of the sheath is adjacent to the head, only separated from it by a small collar, which is not obvious in the other micrographs so far mentioned. It appears from Pl. 2, figs. 12, 13, and other micrographs, that the contracted sheath can take up any position on the core. This sheath also shows two dark lines running down its length; these are clearer in Pl. 2, fig. 13. A headless tail with contracted sheath is shown in Pl. 2, fig. 14; two small discs can be seen at the top of the core where it would be joined to the head. These probably represent a 'double washer' arrangement by which the massive tail is firmly attached to the head; this feature has only previously been found on two phages, one specific to *Escherichia coli* (Bradley, 1964) and one to *Pseudomonas syringae* (Matthews & Bradley, 1964).

In Pl. 2, fig. 15, the major portion of a tail core with the head still attached is shown. The head protein seems to have been slightly degraded, revealing the 'double washer' arrangement clearly. It would appear from this micrograph that the protein plug in Pl. 1, fig. 7, is in fact the inner 'washer'.

The use of the spot test and other plating methods described above indicated that killer particle GA-2 was able only to kill sensitive host bacteria and not to multiply within them. Particles of this morphology were found in large numbers in extracts of plates of *Bacillus subtilis* and *B. licheniformis*, presumably being spontaneously released as is the case with most temperate phages; there was no need to induce them with ultraviolet radiation, antibiotics or hydrogen peroxide. Other particles of varied morphology (Pl. 2, fig. 16) were found similarly associated with the Bacillus species isolated from rotting grass, but were not examined in detail.

Bacillus subtilis phage GF-2

Several phage isolates with a conventional contractile tail were found associated with another strain of *Bacillus subtilis*; one of them (GF-2) was retained. A micrograph of an intact virion is shown in Pl. 3, fig. 17. The head is hexagonal and about 1150 Å in mean diameter. The rather thin tail is 2800 Å long and has a base-plate but no obvious tail fibres or pins. A head is shown in Pl. 3, fig. 20, which looks too complex to be an octahedron but might well be an icosahedron. The tail can contract in two ways: it either decreases its length by one half (Pl. 3, fig. 18), a state of affairs observed after storage for some months, or by about one quarter (Pl. 3, fig. 19). The latter state occurred spontaneously on the preparation of a fresh lysate. The amount of contraction associated with adsorption is not known for certain, but is probably that shown in Pl. 3, fig. 18, resembling other contractile phages.

Acetobacter phage AA-1

The unusual morphology of this Acetobacter phage is shown in Pl. 3, fig. 21, at low magnification; it is not unlike coliphage T3, but differs in the tail structure, which consists of three prongs when seen in profile. Also the head capsomeres are clearly visible though it is not possible to see how they are arranged. The capsomeres and the hexagonal outline of the head are clearer in Pl. 3, fig. 22. The head size is about 650 Å, near to that of coliphage T3. The tail is shown better in Pl. 3, fig. 23. It is not unlike the base-plate of T-even coliphages, but is smaller, the total length being only 185 Å, and the prongs 130 Å. The head capsomeres in this figure show as apparent breaks in the protein coat with a spacing of about 130 Å. In the empty virion in Pl. 3, fig. 24, a different picture is revealed. There are only two tail prongs, and the head capsomeres appear as hollow circular units at a spacing of only 45 Å. However, the coarse 130 Å structure is again apparent in the capsids in Pl. 3, fig. 25. These different appearances present a problem in interpretation which will be discussed below. A contaminant phage was also found in a preparation of Acetobacter phage AA-1; this is seen in Pl. 3, fig. 26, and shows that other morphological types may be found in rotting apples. This contaminant resembles some Staphylococcus phages (Bradley, 1963*a*) and so might be presumed to come from a related genus, perhaps *Micrococcus*.

DISCUSSION

The virulent Bacillus subtilis phages. The new morphological type, Bacillus phage GA-1, which appears to be common in nature, is the most important of the two described here. It belongs to the morphological group of phages with short non-contractile tails (Bradley, 1965*a*) and is closest to coliphage P22 (Anderson, 1960). Although one cannot be certain from the electron micrographs shown about the true shape of phage GA-1, by assuming that the rules of symmetry are obeyed (Bradley, 1963*a*, *b*) certain deductions can be made. To ascertain the head shape, the radial symmetry of the tail must first be determined. The micrographs show a tail plate with three or four visible pins or subunits, and this strongly suggests a sixfold rather than a fourfold or fivefold radial symmetry. The base-plate of T-even coliphages, with its six pins, usually shows only three in profile in an electron micrograph, a similar state of affairs. Thus, with the tail probably having a sixfold radial symmetry, the long axis of the head to which it is attached will be the same. This, when considered in conjunction with the outline of the head perpendicular to the long axis, as seen in electron micrographs, suggests a bipyramidal hexagonal prism. It is to be noted that the T-even coliphage head was thought to be this shape for what were basically the same reasons, but facets visible on electron

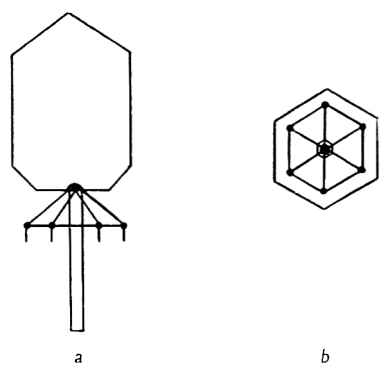

Fig. 1. Diagram of Bacillus phage GA-1; (*a*) side view; (*b*) view down axis of tail, ×350,000.

micrographs showed the assumption to be incorrect (Bradley, 1965*b*). No facets can be seen here, but the head outline is quite different to that of the T-even coliphages, which are much fatter—i.e. they have a larger width/length ratio. It seems not unlikely that Bacillus phage GA-1, with its more slender appearance, shows the true outline of a bipyramidal hexagonal prism. The Staphylococcus phage 594n (Bradley, 1963*a*) has a similarly shaped head. Unfortunately it is likely to be very difficult to demonstrate a bipyramidal hexagonal prism conclusively in the electron microscope. These interpretations are illustrated diagrammatically in Fig. 1. Bacillus phage GF-2 is described here because it again raises the question of head shape. Phages with heads more than 400 Å in size seem to prefer the octahedral form, though there are exceptions. One of these (SP8), specific to *Bacillus subtilis*, was described by Davison (1963); it resembles Bacillus phage GF-2 though the tail is much shorter; convincing evidence is shown to indicate that SP8 is an icosa-

hedron. A similar isolate (SP50) was described by Eiserling & Boy de la Tour (1965), who stated that this phage head is also probably an icosahedron, though their micrographs suggest that an octahedron is more likely. However, the tail plate of SP50 has five distinct pins, which also appears to be the case with SP8. Thus, in the case of SP8 the symmetry rule is obeyed, and there is no reason to suppose that SP50 is an exception. With Bacillus phage GF-2, there are no tail pins to act as a guide to head shape, but from Pl. 3, fig. 20, and the two precedents it also seems likely to have an icosahedral head. From these observations it is important to note that an electron micrograph of an icosahedral body can often appear very like one of an octahedral body; care needs to be taken in the interpretation (Bradley, 1963*b*).

These Bacillus phages are thus further examples of virions which conform to the symmetry rule. It is becoming increasingly necessary to establish the extent to which this rule is obeyed since the validity of various recent important interpretations of the T-even coliphage head shape may depend upon it (Bradley, 1965*b*; Moody, 1965; Boy de la Tour & Kellenberger, 1965).

Bacillus killer particles. There have been several reports of the isolation of killer particles from strains of *Bacillus subtilis* and *B. licheniformis*, most notably by Seaman *et al.* (1964) and Stickler, Tucker & Kay (1965). These authors reported that the particles were only obtained after induction of the host strain with mitomycin C and with hydrogen peroxide, respectively. The particle GA-2, on the other hand, was released spontaneously from both these species under normal growth conditions. Perhaps because of its unusual appearance, the GA-2 morphological form was studied in greatest detail by the above authors. The electron micrographs of Stickler *et al.* (1965) give an opportunity for comparison, and the only real differences appear to be in the length of the tail, and hence the number of striations. According to Stickler *et al.* (1965) there were six tail fibres, but, from the observations given here, five would appear more likely. If the symmetry rule holds, this would conveniently fit the fivefold axis of an icosahedral head, a form favoured by small viral capsids. The tail capsomeres are particularly interesting. They appear circular in some places (Pl. 2, fig. 10, arrowed), indicating that they are tubular. The results of acridine orange staining (Bradley, 1965*c*) suggest that the head contains double-stranded DNA like the other killer particles mentioned.

Acetobacter phage AA-1. It is difficult to obtain electron micrographs which clearly demonstrate the shape of this phage but again, assuming the symmetry rule, a probable form can be deduced from the tail. The appearance of three or two prongs strongly indicates a fourfold radial symmetry, which would make the head octahedral. The number of visible prongs would depend upon the orientation of a square plate having one at each corner. Judging from the apparent size of the head capsomeres in Pl. 3, figs. 21–23, one would expect to see their arrangement clearly, but the empty virion seems to have much smaller ones. It is considered that the large capsomeres are really an artifact produced by the superposition of the regular array of smaller ones on each side of the head; it must be remembered in interpreting electron micrographs of negatively stained virions that contrast is produced by portions of the viral surface adjacent to as well as away from the specimen support film.

Conclusion. It seems likely from the foregoing that the examination of bacterio-

phages and similar particles as they occur in nature is likely to produce interesting material for physiological, chemical and genetic studies. The Bacillus killer particles described appear to lie somewhere between the non-infective virus-like rods of *Saprospira grandis* (Correll & Lewin, 1964) and a true temperate phage. It is interesting to list the various particles found associated with bacteria. There are virulent phages with ability to multiply within the host and temperate phages, many of which behave in a virulent fashion towards sensitive hosts. The killer particles are produced from the host in the same way as temperate phages, but are unable to act in a virulent fashion, or, in the case of GA-2, to lysogenize a sensitive host. The bacteriocins resemble the killer particles in that they cannot multiply. When one compares the properties of bacteriocins with those of the killer particles one finds such a close resemblance that it seems possible that many if not all of those so far discovered are defective phages. This is contrary to the views expressed by Jacob & Wollman in 1959, but killer particles had not at that time been discovered. Further evidence to support this has been provided by Sandoval, Reilly & Tandler (1965), who showed that colicin 15 is probably a killer particle. Finally there are the non-infective rods of *Saprospira grandis*, again released spontaneously like Bacillus killer particle GA-2. Each of the members in this list is linked in some physiological characteristic and the continued study of their interrelationships may provide information on the question of the origin of bacteriophages and hence of viruses in general.

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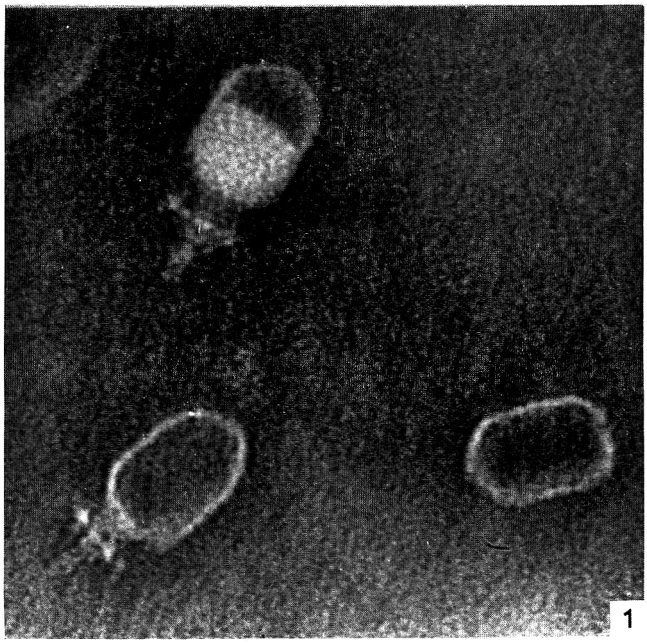

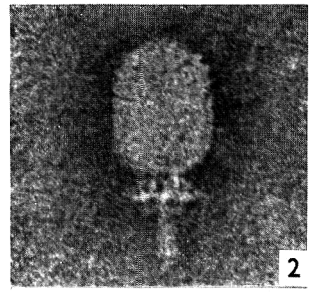

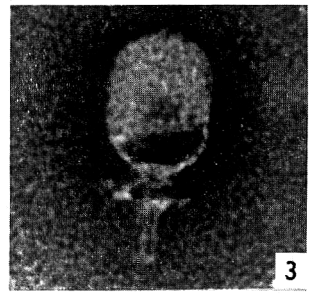

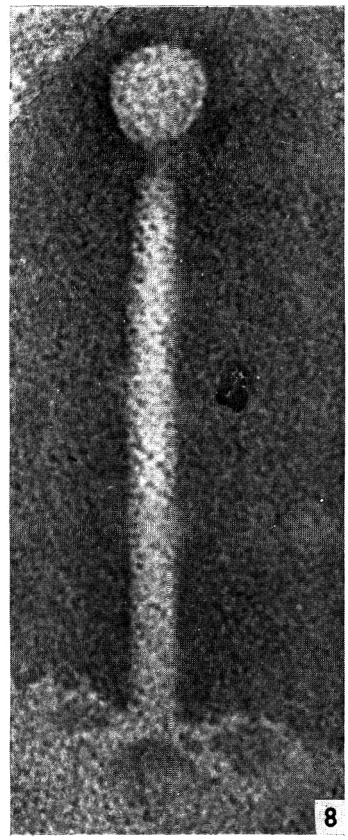

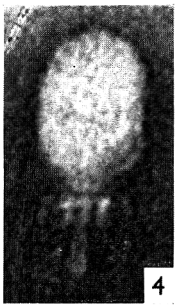

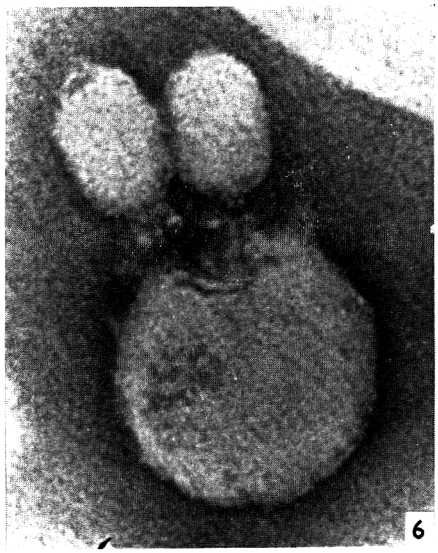

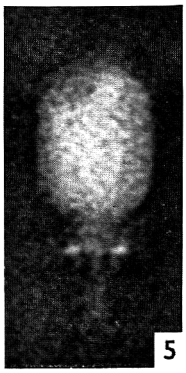

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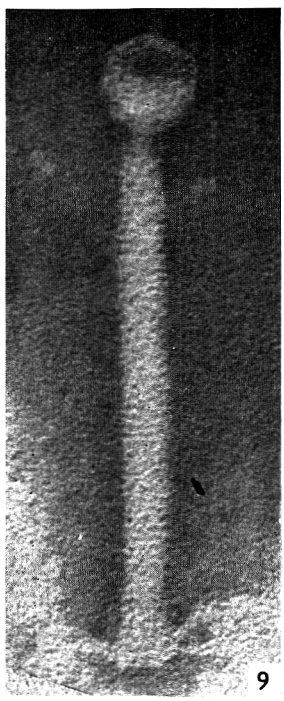
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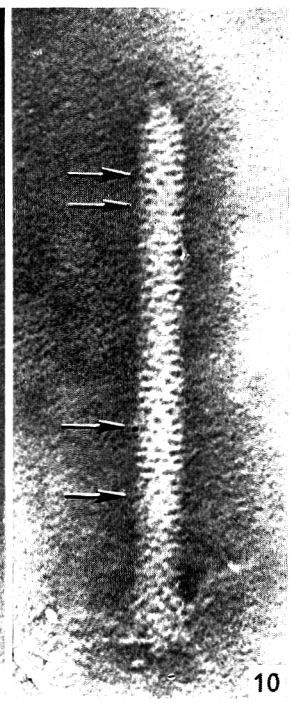
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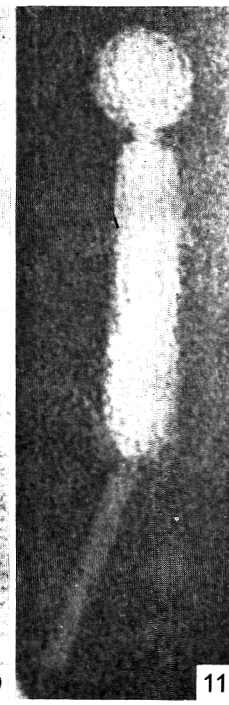
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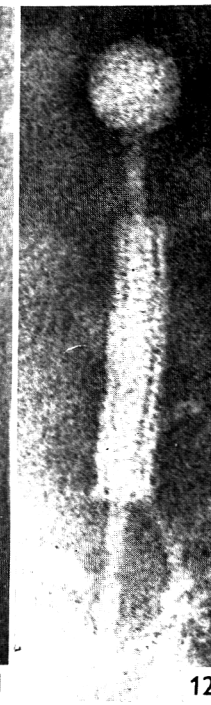
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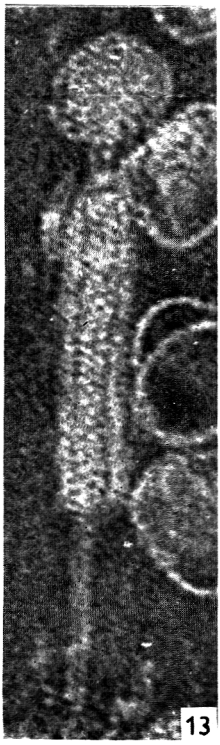
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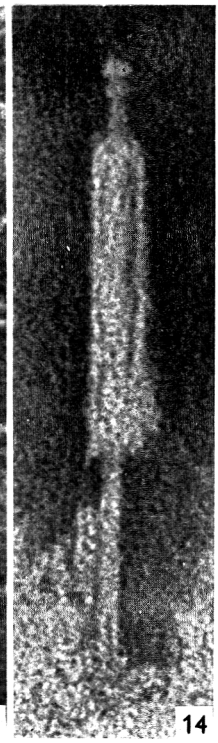
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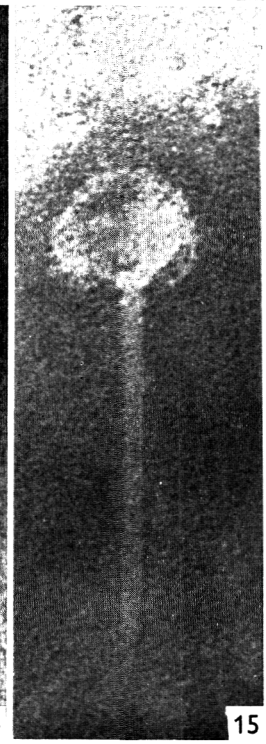
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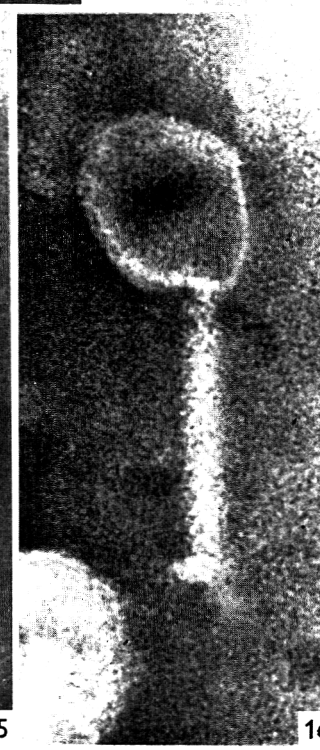
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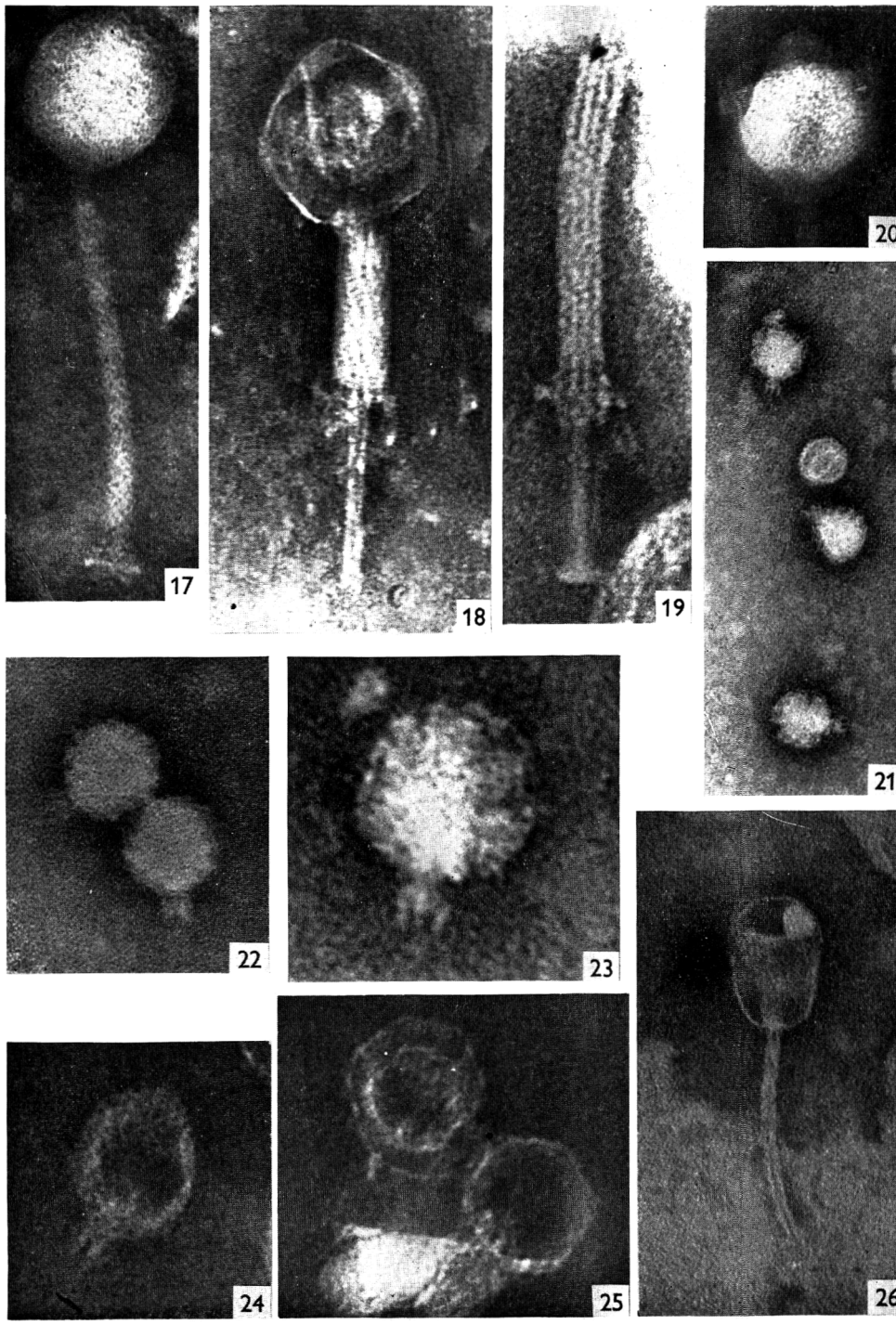

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–5. Phage GA-1, × 333,000.

Fig. 6. Phage GA-1 particles adsorbed to debris, × 333,000.

Fig. 7. Empty head and part of tail core of killer particle GA-2, × 333,000.

Fig. 8. Intact killer particle GA-2, × 333,000.

PLATE 2

Fig. 9. Killer particle GA-2 showing partly empty head with hexagonal outline, × 333,000.

Fig. 10. Headless tail of GA-2 showing tail capsomeres, × 333,000.

Figs. 11–13. GA-2 killer particles with contracted tail sheaths, × 333,000.

Fig. 14. Headless GA-2 particle with double washer, × 333,000.

Fig. 15. Sheathless GA-2 particle showing core and double washer, × 333,000.

Fig. 16. Another morphological type of killer particle, × 333,000.

PLATE 3

Fig. 17. Intact GF-2 virion, × 200,000.

Fig. 18. GF-2 with fully contracted sheath, × 200,000.

Fig. 19. Partly contracted GF-2 sheath, × 333,000.

Fig. 20. GF-2 head showing numerous facets, × 200,000.

Fig. 21. Intact virions of phage AA-1, × 110,000.

Fig. 22. Phage AA-1 virions showing head capsomeres, × 220,000.

Fig. 23. Virion of phage AA-1 showing three-pronged tail, × 380,000.

Fig. 24. Empty virion of AA-1 with two-pronged tail, × 333,000.

Fig. 25. Empty virions of AA-1, × 333,000.

Fig. 26. Particle of a phage from rotten apple, × 200,000.

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Composition of Cell Walls of Some Gram-Negative Cocci

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SUMMARY

Cell walls isolated from six species of *Neisseria* contained 17 amino acids, 6·3–8·3% mucopeptide, and 11·4–30·0% lipid. *Veillonella parvula* cell walls were similar, differing principally in the presence of 24·5% mucopeptide. The mucopeptide of all seven Gram-negative cocci contained glucosamine, muramic acid, glutamic acid, alanine, glycine and DL-diaminopimelic acid as principal components; the mucopeptide from *V. parvula* cell walls also contained an unidentified ninhydrin-reacting component. The marked similarity of the cell walls of the Gram-negative cocci and those of Gram-negative bacilli indicates a closer relationship between these two groups than between the Gram-negative and Gram-positive cocci.

INTRODUCTION

On the basis of progressive changes in several properties, Bisset (1959) arranged the following genera of Gram-positive and Gram-negative cocci in a supposed phylogenetic series which started with *Sarcina*, passed through *Micrococcus* and *Staphylococcus* to *Neisseria*, and terminated with *Veillonella*. In an attempt to avoid relating two such distinct groups as the Gram-positive and Gram-negative bacteria, he claimed that *Neisseria* and *Veillonella* were only superficially Gram-negative (Bisset, 1959, 1962). In view of the marked chemical and structural differences between the cell walls of Gram-positive and Gram-negative bacteria (Salton, 1960; see also Murray, 1962; Perkins, 1963) Bisset's claim implies that the cell walls of *Neisseria* and *Veillonella* would be of the Gram-positive type. However, apart from data on the presence of lipid and amino sugar in the cell walls of *Neisseria catarrhalis* (Salton, 1963), little information is available on the chemical composition of the cell walls of Gram-negative cocci. We therefore examined some of the more important components of cell walls isolated from *Veillonella parvula* and six species of *Neisseria*.

METHODS

Organisms and growth conditions. *Veillonella parvula* no. 10,790 and *Sarcina lutea* no. 9341 were obtained from the American Type Culture Collection. *Neisseria animalis* no. 10,212, *N. catarrhalis* no. 3622, *N. caviae* no. 10,293, *N. flavescens* no. 8263, *N. pharyngis* no. 4591, *N. pharyngis-flavus* no. 4590, *Staphylococcus saprophyticus* no. 7291 and *Micrococcus lysodeikticus* no. 2665 were obtained from the National Collection of Type Cultures (Colindale, London). *Escherichia coli* CL 30 was obtained from Dr B. A. D. Stocker and *E. coli* 173-25, auxotrophic for LL- or DL-diaminopimelic acid (Davis, 1952; Hoare & Work, 1955), from Dr J. Leder-

berg. The bacteria were stored as lyophilized cultures (Annear, 1962) and maintained on meat infusion agar (*Mackie and McCartney's Handbook*, 1960) supplemented, where necessary, with 10% (v/v) defibrinated horse blood or diaminopimelic acid 10 μg./ml.

Veillonella parvula was grown anaerobically (McIntosh and Fildes jar) at 37° for 18–24 hr in 100 ml. batches of a modification of the medium of Rogosa (1956) which contained per litre: Trypticase (Baltimore Biological Laboratory Inc.), 10 g.; yeast extract (Baltimore Biological Laboratory Inc.), 5 g.; sodium lactate (85%), 10 g. All other bacteria were grown at 37° for 18–24 hr in 400 ml. batches of meat infusion broth (supplemented with 10% (v/v) horse serum for *Neisseria animalis*, *N. pharyngis* and *N. pharyngis-flavus*) in 1-l. Erlenmeyer flasks on a reciprocal shaker (Kantorowicz, 1951).

Isolation of cell walls. Bacteria were harvested in a refrigerated centrifuge and washed three times with cold distilled water. Portions (5–10 g.) of packed wet bacteria and 10–25 g. glass beads (Ballotini no. 12) were placed in the capsule of a cell disintegrator (Nossal, 1953) and shaken for three 1 min. periods, the capsule being cooled in ice-water between runs. Phase-contrast microscopy showed that almost all the bacteria were broken by this treatment. The capsule was washed out with 50 ml. cold distilled water and the supernatant fluid decanted after the glass beads had settled. The beads were washed three times with distilled water and the combined washings centrifuged at 2000*g* for 20 min. to remove unbroken bacteria, steel particles and other debris. The supernatant fluid was centrifuged at 20,000*g* for 15 min. and the deposited cell walls washed three times with distilled water, frozen and dried *in vacuo* over P_2O_5.

Preparation of mucopeptide fraction. The mucopeptide fraction of the cell wall was isolated by a modification of the method of Schocher, Bayley & Watson (1962). Lyophilized cell walls were suspended in 20% (w/v) sodium dodecyl sulphate and incubated at 37° for 18 hr, with gentle shaking. The suspension was centrifuged at 20,000*g* for 30 min., and the deposited mucopeptide fraction freed from sodium dodecyl sulphate by washing with distilled water at least five times; the fraction was then frozen and dried *in vacuo* over P_2O_5.

Analytical methods

Sodium dodecyl sulphate was estimated spectrophotometrically by the method of Graham & Whitney (1959).

Paper chromatography was used to determine the amino acids and amino sugars present in acid-hydrolysed cell walls and mucopeptide fractions. Hydrolysis was done in sealed tubes with 6 N-HCl at 105° for 18 hr; subsequently the HCl was removed *in vacuo* over solid NaOH and P_2O_5 at 37°, and the dried residue dissolved in distilled water. Single-dimensional descending chromatography on Whatman no. 4 paper was done with the following solvent systems: (A) *n*-butanol + acetic acid + water (12 + 3 + 5, by vol.); (B) *n*-butanol + pyridine + water + acetic acid (60 + 40 + 30 + 3, by vol.; Primosigh, Pelzer, Maass & Weidel, 1961); (C) methanol + water + 10 N-HCl + pyridine (80 + 17·5 + 2·5 + 10, by vol.; Rhuland, Work, Denman & Hoare, 1955). Two-dimensional ascending chromatography was done on Whatman no. 1 paper with methanol + water + pyridine (80 + 20 + 4, by vol.) as the first solvent and *tert.*-butanol + methylethylketone + water + diethylamine (40 + 40 +

20 + 4, by vol.) as the second solvent (Redfield, 1953). Amino acids and amino sugars were detected by spraying with ninhydrin (0·2%, w/v, in acetone) and heating at 100° for 3 min. Amino sugars were confirmed with a modified Elson–Morgan reagent (Smith, 1958) and alkaline silver nitrate (Smith, 1958).

Isomers of diaminopimelic acid were identified by chromatography in solvent C (Rhuland *et al.* 1955), and by the growth response of *Escherichia coli* 173-25 to the diaminopimelic acid contained in discs cut from unstained strips of chromatograms developed with solvent A.

Total lipid was determined by ether extraction of cell-wall preparations after hydrolysis in 6 N-HCl for 4 hr at 100° (Salton, 1953).

RESULTS

In addition to the cell walls of the seven species of Gram-negative cocci, we also examined, for reference purposes, the cell walls of three species of Gram-positive cocci and a typical Gram-negative bacterium.

The mucopeptide content for each of the eleven species of bacteria is shown in Table 1. The walls of the six species of *Neisseria* gave an average mucopeptide yield of 6·9%; a value very close to that obtained for the *Escherichia coli* CL 30. In comparison, *Veillonella parvula* cell walls contained 24·5% mucopeptide, that is about 3·5 times more than the other Gram-negative bacteria; nevertheless, this corresponded to only about one-third of the amount found in the three Gram-positive cocci. (The estimate of 24·5% mucopeptide in cell walls of *V. parvula* was obtained by pooling the results of two independent experiments which gave 23·1% and 26·4% mucopeptide, respectively; a total of 143·4 mg. dry weight cell wall were analysed.) However, there are two possible sources of error in the determination of the proportion of mucopeptide in the cell wall: (i) adsorption of sodium dodecyl sulphate on to the mucopeptide (Putnam, 1948); (ii) loss of mucopeptide during the repeated washings following sodium dodecyl sulphate treatment of cell walls. Examination of cell walls of *Micrococcus lysodeikticus* following sodium dodecyl sulphate treatment and subsequent washings revealed no detectable (less than 0·5%) bound sodium dodecyl sulphate; on the other hand, an incubation and washing régime equivalent to that used for the isolation of mucopeptide but, with the sodium dodecyl sulphate omitted, resulted in losses of up to 15% of cell-wall preparations of *Neisseria animalis*, *N. caviae* and *Staphylococcus saprophyticus*. Thus, because of losses during washing, our estimates for the proportion of mucopeptide in cell walls are probably less than the true values.

Chromatography of acid hydrolysates of cell walls of *Veillonella parvula*, the six Neisseria species and of *Escherichia coli* showed the following amino acids and amino sugars: diaminopimelic acid, arginine, lysine, aspartic acid, glutamic acid, histidine (trace), glycine, serine, alanine, proline, tyrosine, valine, methionine (trace), isoleucine, leucine, phenylalanine, threonine, muramic acid, glucosamine (see Table 1). Cell walls of *V. parvula* also contained an unidentified ninhydrin-reacting constituent; this material, like diaminopimelic acid, was not always obvious in chromatograms of cell-wall hydrolysates because of its relatively low concentration. The mucopeptide isolated from cell walls of the six Neisseria species and of *Escherichia coli* contained diaminopimelic acid, alanine, glutamic

Table 1. *Composition of cell walls and mucopeptide fraction of Gram-negative and Gram-positive bacteria*

Bacteria	No. of amino acids in cell walls	Mucopeptide (% of dry wt. of cell wall)	Principal amino acids present in mucopeptide					Total lipid (% of dry wt. of cell wall)
			Glutamic acid	Alanine	Lysine	Diamino-pimelic acid	Glycine	
Gram-negative								
Escherichia coli	17	7·2 (103·1)†	+	+	−	+	−	15·6 (86·2)†
Neisseria animalis	17	6·4 (58·2)	+	+	−	+	−	16·8 (163·6)
N. caviae	17	6·4 (273·0)	+	+	−	+	−	30·0 (67·0)
N. catarrhalis	17	7·8 (127·6)	+	+	−	+	−	.
N. flavescens	17	6·8 (87·8)	+	+	−	+	−	.
N. pharyngis	17	6·6 (56·4)	+	+	−	+	−	.
N. pharyngis-flavus	17	8·3 (87·4)	+	+	−	+	−	11·4 (115·7)
Veillonella parvula	17*	24·5 (143·4)	+	+	−	+	−	27·0 (59·0)
Gram-positive								
Staphylococcus saprophyticus	4	73·5 (37·4)	+	+	+	−	+	0 (58·0)
Micrococcus lysodeikticus	4	90·8 (93·5)	+	+	+	−	+	.
Sarcina lutea	3	68·0 (29·4)	+	+	+	−	−	.

* An unidentified ninhydrin-reacting component was also present in the cell wall and mucopeptide of *Veillonella parvula*.

† The figures in parentheses show the mg. dry wt. cell wall analysed; . = not done.

acid, muramic acid and glucosamine as principal components (see Table 1); all other amino acids were either absent or present only in trace amounts. In addition to the amino acids and amino sugars found in the mucopeptide of *E. coli* and the neisserias, *V. parvula* also contained the previously mentioned unidentified ninhydrin-reacting component now present in relatively high concentration. The unknown component formed a single spot in single-dimensional chromatograms, but in the two-dimensional system, although it remained as a single spot at the origin after development with the first solvent, two spots (R_F values 0·05 and 0·13, respectively) were observed with the second solvent.

As expected, treatment with sodium dodecyl sulphate produced no obvious qualitative changes in the composition of the cell walls of the three Gram-positive cocci. The principal components detected in the cell walls of these three cocci were lysine, glutamic acid, alanine, muramic acid and glucosamine; the walls of *Staphylococcus saprophyticus* and *Micrococcus lysodeikticus* also contained glycine (see Table 1), and there were trace amounts of serine and phenylalanine in *S. saprophyticus*.

The diaminopimelic acid from *Veillonella parvula* and all six Neisseria species showed an R_F value corresponding to the DL or DD isomers in chromatograms developed with solvent C and permitted the growth of *Escherichia coli* 173-25. This indicated the presence of the DL isomer in the walls of all seven Gram-negative cocci examined.

All four species of Gram-negative cocci examined contained considerable amounts of lipid (Table 1): *Neisseria caviae* and *Veillonella parvula* cell walls contained about twice the amount present in cell walls of *Escherichia coli* and the other two neisserias. No lipid was detected in the cell walls of *Staphylococcus saprophyticus*.

DISCUSSION

In general, the cell walls of Gram-positive bacteria are characterized by a high proportion of mucopeptide, the presence of a few amino acids, and a low lipid content; in addition, the majority of such members of the family Micrococcaceae as have been examined contain lysine (and no diaminopimelic acid) and glycine as principal components of the cell-wall mucopeptide. On the other hand, the cell walls of Gram-negative bacteria contain a small amount of mucopeptide, a wide range of amino acids, and a substantial amount of lipid (see Salton, 1960; Perkins, 1963). In respect of the foregoing criteria, our results for *Escherichia coli* and the three species of Gram-positive cocci examined agree with previously published data, except that we were unable to detect glycine in the mucopeptide of the *Sarcina lutea* (Weidel, Frank & Martin, 1960; Perkins, 1963). Further, the cell walls of the six Neisseria species in so far as we have examined them, show no affinity with the cell walls of the Gram-positive cocci, but closely resemble those of the Gram-negative bacteria as typified by *E. coli* (see Table 1). *Veillonella parvula* differed only in having a somewhat higher mucopeptide content, but still well below that commonly found in Gram-positive cocci.

The cell walls of Gram-positive and Gram-negative bacteria are just as distinctive structurally as they are chemically. In thin section, the former exhibit a homogeneous outer layer adhering to the plasma membrane (see Glauert, 1962; Murray,

1962); on the other hand Gram-negative bacteria, including species of *Neisseria* and *Veillonella*, show a corrugated triple-layered membrane overlying what seems to be the rigid layer of the wall (Murray, 1963; Murray, Reyn & Birch-Andersen, 1963; Bladen & Mergenhagen, 1964). As a result of the differences in structural organization of the cell walls, the surface of Gram-positive bacteria appears smooth and that of Gram-negative bacteria markedly rugose (Zwillenberg, 1964); the Veillonella species examined by Bladen & Mergenhagen (1964) showed a rugose surface.

A prominent feature of the sections of the Veillonella examined by Bladen & Mergenhagen (1964) was the unusually thick (70 Å) 'solid membrane' lying between the outer triple-layered membrane and the plasma membrane. If this middle membrane is the rigid mucopeptide layer, as suggested by Bladen & Mergenhagen, then our finding of at least 25% mucopeptide in Veillonella cell walls is not surprising. In view of the comparatively lower mucopeptide content of walls of all six Neisseria species a corresponding decrease in the thickness of the rigid layer would be expected. In fact, the rigid layer in Neisseria appears to be 20–30 Å in thickness (Dr R. G. E. Murray, personal communication).

Thus the previously demonstrated structural relationship between the genera *Veillonella*, and *Neisseria* and other Gram-negative bacteria is complemented by the chemical data obtained in the present work. This relationship is strengthened by the close affinity of the genus *Neisseria* to the rod-shaped Gram-negative bacteria shown in the numerical taxonomic survey of Sneath & Cowan (1958). Collectively, these observations would appear to refute Bisset's proposal for the derivation of the Gram-negative cocci from their Gram-positive counterparts (Bisset, 1959, 1962). Indeed, Veillonella, with a relatively thick rigid layer and the ability under certain conditions to divest itself of its outer triple-layered membrane and so structurally mimic the Gram-positive cocci (Bladen & Mergenhagen, 1964), could represent a transitional stage in the development of the typically Gram-positive cocci from the typically Gram-negative cocci. Alternatively, the gross morphological resemblance between the Gram-negative and Gram-positive cocci could be the result of convergent evolution rather than direct derivation.

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Electron Microscopy Studies on the Immobilization Antigens of *Paramecium aurelia*

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SUMMARY

The immobilization antigens of *Paramecium aurelia* were located in the electron microscope by the use of antibody labelled with electron-dense ferritin. On treatment of fixed whole paramecia with ferritin-labelled antibody, followed by sectioning, ferritin granules were seen on the pellicle and cilia of homologous organisms only. By counterstaining the sections with an electron-dense stain (potassium permanganate+uranyl acetate) the absorbed globulin of the antibody was revealed as a thick fuzz on pellicle and cilia. Antigen was present over the entire surface of the organism with the exception of the gullet area. Transformation from one antigenic type to another, by change of temperature, showed the 'new' antigen to appear initially on the pellicle and subsequently on cilia. The ferritin labelling, with counterstaining, proved to be very sensitive; ferritin granules and globulin fuzz denoting 'new' antigen appearance were detected at isolated sites on the pellicle and cilia after only 1 hr of growth at the higher temperature. The number of these sites then increased, until the granules and fuzz covered the entire surface of first the pellicle and subsequently cilia. Attempts to study the internal antigens with ferritin-labelled antibody were not successful.

INTRODUCTION

In previous papers the immobilization antigens of Paramecium were located by the use of fluorescent-labelled specific antibodies for examination under ultraviolet radiation (Coons, 1956). In the first application of this fluorescence technique to paramecium antigens (Beale & Kacser, 1957), whole paramecia, both living and fixed, were treated with fluorescein-labelled antisera, and the immobilization antigens were shown to be present on the pellicle and cilia, with the exception of the gullet area. Later (Beale & Mott, 1962) the interior of Paramecium was studied by treating sections of fixed organisms with antibody conjugated with either fluorescein or rhodamine. Fluorescence was observed on pellicle and cilia, and in the internal cytoplasm with the exception of the macronucleus. By pre-treating the sections with heterologous non-fluorescent antiserum, no fluorescence was observed in the cytoplasm, and fluorescence occurred only on the pellicle and cilia of homologous organisms. It was concluded that the immobilization antigens are located on the pellicle and cilia, and that the internal cytoplasmic antigens are relatively invariant in paramecia of different immobilization antigenic type. Transformation from one antigenic type to another by change of temperature, observed by using the technique of double labelling with fluorescein+rhodamine-labelled antisera, showed the

'new' antigen to appear initially on the pellicle and subsequently on the cilia. Higher magnifications were necessary, however, to study the exact location of the immobilization antigen and other details of the membranes on which the antigen was thought to be located. This present report, therefore, covers the use of the electron microscope and ferritin, a molecule with an electron-dense core of iron surrounded by a protein shell, as the antibody level (Singer, 1959).

METHODS

The general methods used were those published by Beale & Mott (1962).

Stocks and serotypes of Paramecium aurelia. Stock 168, variety 1, serotypes G and D, were used. Type 168G is stable at 25°, type 168D at temperature above 30°. Transformation from type 168G to type 168D was obtained as in Beale & Mott (1962). Samples of paramecia were taken at intervals and examined for the stage of antigenic change by three methods: (i) fixed paramecia were studied with the electron microscope and ferritin labelling; (ii) with the light microscope and fluorescent labelling, while at the same time, (iii), live samples were tested for immobilization with antisera to the initial and final antigens.

General cultivation techniques. The paramecia were grown according to the general methods of Sonneborn (1950), with dried lettuce leaf infusions and with *Aerobacter aerogenes* at pH 6·8–7·4; this gave about 5 fissions in 24 hr.

Maintenance solution. A non-nutrient medium was used for washing the paramecia; it consisted of 0·013M-NaCl, 0·0003M-KCl, 0·0003M-$CaCl_2$, 0·004M-NaK phosphate buffer; pH 7. (The molarity of the $CaCl_2$ solution was in error in Beale & Mott, 1962.)

Preparation of antisera. Antisera were made against whole paramecia according to the method of Sonneborn (1950) by injecting homogenates of whole organisms into rabbits. The sera were incubated at 56° to inactivate complement, dialysed against Maintenance Solution and stored frozen in small samples. Antisera were developed against antigens 168D and 168G; and a 'heterologous' antiserum, i.e. one not reacting with the immobilization antigens 168D and 168G, was developed against stock 192X.

Preparation of fluorescent labelled antibody. A modification of the method of Coons & Kaplan (1950) by Marshall, Eveland & Smith (1958) was used to label antibody with fluorescein; the method of Chadwick, McEntegart & Nairn (1958) was used to label antibody with rhodamine, as in Beale & Mott (1962).

A preliminary brief account of the following methods was published by Mott (1963).

Preparation of ferritin-labelled γ-globulin. The γ-globulins of the antisera were precipitated by half-saturation with ammonium sulphate, the final precipitated globulins being made up in and dialysed against Maintenance Solution, to a final concentration of 20 mg. γ-globulin/ml. Some of this globulin from each antiserum was retained unlabelled, and the remainder labelled with ferritin. Ferritin preparations from two sources were used: the first, prepared by Dr P. M. Harrison (Department of Biochemistry, The University, Sheffield), was obtained from Dr R. M. Clayton; the second was obtained from Calbiochem, Los Angeles, California, U.S.A., as a preparation in $CdSO_4$, crystallized × 6. Ferritin was coupled to the γ-globulin by

using the *m*-xylylene di-isocyanate bifunctional coupling agent of Singer (1959) in the improved method of Singer & Schick (1961). The ferritin-labelled globulin was then dialysed against Maintenance Solution and centrifuged at 10,000 rev./min. for 30 min. This gave a clear stable solution; small samples were stored frozen at −20°. The loss of activity of the globulin during the conjugation procedure was about 30%.

Treatment with antibody. Paramecia were gently centrifuged down, washed in Maintenance Solution, and then fixed for 30 min., with occasional mixing, in 1% (w/v) osmic acid in 0·014 M-veronal acetate buffer of pH 7·4; (Palade, 1952) containing 1·3% sucrose (Caulfield, 1957). The fixed organisms were pre-treated first with unlabelled, heterologous (anti-192X) serum, diluted 1/2, for 1 hr as with the fluorescence technique, and then treated with the specific ferritin-labelled γ-globulin, undiluted, for 1–2 hr. The organisms were well washed in Maintenance Solution between procedures.

Araldite embedding and sectioning. The paramecia were dehydrated in increasing ethanol concentrations and embedded in the epoxy-resin Araldite M. (Glauert & Glauert, 1958) using volatile 1–2 epoxy propane with the Araldite for better penetration (Luft, 1961). Sections were cut on a Porter-Blum microtome with glass knives, and were examined on grids with a supporting film of formvar and carbon.

Counterstaining the sections. Some grids were left unstained to demonstrate ferritin granules in the sections. Other sections were counterstained by floating the grids, section downwards, on a solution containing potassium permanganate and uranyl acetate, a modification of methods by Lawn (1960) and Watson (1958). The grids were floated for 20 min. on 2·5% (w/v) uranyl acetate in 1% (w/v) potassium permanganate, and washed by floating on the surface of distilled water. The sections were then partly decolorized by floating, for 30 sec. only, on a dilute citric acid solution (about 1 ml. of 5%, w/v, citric acid + 30 ml. distilled water), then again washed with distilled water.

Electron microscopy. The sections were examined in an electron microscope, using either a Philips E.M. 75 model with plate magnifications up to × 12,000, or a Siemens Elmiskop-I model, generally with a plate magnification of × 30,000.

RESULTS

The specificity of the labelling techniques and the location of antigen

The osmic acid fixation did not result in loss of antigenic activity, in agreement with the earlier findings with paramecium antigens by Beale & Kacser (1957) and with other antigens by Spendlove & Singer (1961). Fixed whole paramecia of two different serotypes, 168G and 168D, were separately treated with ferritin-labelled anti-168D γ-globulin, after pre-treatment with heterologous unlabelled antiserum as described in Methods. After embedding in Araldite, sectioning and viewing the unstained sections under the electron microscope, ferritin granules were seen on the pellicle and cilia of homologous organisms (Pl. 1, fig. 1) but not on the non-homologous organisms. The ferritin granules (diameter about 50 Å) were only seen at high magnifications, but on counterstaining the sections with uranyl acetate + potassium permanganate, the absorbed globulin of the specific antibody was revealed even at low magnifications as a thick fuzz which covered the pellicle and cilia of homologous

organisms. The conjugated ferritin granules were seen within this fuzz at higher magnifications, no comparable amount of fuzz being present on non-homologous organisms. This globulin fuzz was therefore of use in quickly scanning preparations at low magnifications. These findings were in accordance with those illustrated in Mott (1963).

The absence of ferritin granules and globulin fuzz from the control paramecia established the use of the technique of ferritin labelling and counterstaining for demonstrating the presence of antigen and specific antibody. By examination of many electron micrographs of the surface of paramecia by the above techniques, the immobilization antigen was found to be present over the surface of the pellicle and cilia, with the exception of the gullet area. Here the pellicle and cilia were found to have no fuzz or ferritin granules when the paramecium was treated with ferritin-labelled antibody homologous to the immobilization antigen on the rest of the organism (Pl. 1, fig. 2). It appears there are no antigens other than the immobilization antigens on the surface of paramecia, and that these cover the entire surface with the exception of the gullet area. This agreed with the findings obtained with the fluorescence technique, when paramecia treated with fluorescent antibodies to all the possible antigens (immobilizing antigens and others) showed complete lack of fluorescence in the gullet area (Beale & Kacser, 1957).

Transformation experiment type G → type D

The results of taking samples of organisms at intervals and studying them simultaneously for antigenic change from type 168G to type 168D by the three series of tests (ferritin labelling, fluorescent labelling, immobilization) are shown in Table 1.

From the electron microscope study, samples of paramecia taken at the beginning of the experiment (i.e. at 'nil hours' growth at 35°) showed no ferritin granules or globulin fuzz, following treatment with ferritin-labelled anti-168D γ-globulin (Pl. 2, fig. 3). After growth at 35° for only 1 hr, globulin fuzz and ferritin granules were located initially at isolated sites on the pellicle and at the proximal ends of cilia (Pl. 2, fig. 4). At this stage neither the fluorescence nor the immobilization tests gave any indication of the appearance of new antigen. By examination with the electron microscope it was seen that the antigen sites continued to increase on pellicle and cilia, and were fairly numerous after growth for 3 hr at 35°. At this stage the fluorescence test was just giving faint indication of the appearance of the 'new' antigen (168D) on the pellicle only, while the immobilization tests still gave practically negative results. After growth for 6 hr at 35°, the electron microscope showed many sites of 'new' antigen formation on pellicle and cilia, the fluorescence technique showed a fairly strongly fluorescent pellicle and faintly fluorescent cilia, and the immobilization tests now showed a very slight effect (Pl. 3, fig. 5). An organism examined under the electron microscope after 7 hr at 35° showed that the antigen 168D was distributed homogeneously over the pellicle, but only at isolated sites on the cilia (Pl. 3, fig. 6). At this stage the fluorescence technique showed a very strongly fluorescent pellicle and faintly fluorescent cilia, and the immobilization tests began to show some definite effect. At the final stage (18–24 hr), the fuzz and ferritin granules were seen by electron microscopy, to occur homogeneously over the entire surface of pellicle and cilia, giving the same picture as seen previously with a com-

pletely homologous organism. Both the fluorescence and immobilization tests also showed complete change to the 'new' antigenic types.

The counterstaining of absorbed globulin proved very useful in the study of transformation of the antigens, for when transforming organisms were treated with ferritin-conjugated antibody to the 'new' antigen being formed, the successive stages could be quickly scanned for the first appearance of the globulin fuzz. This was especially so in the early stages, when little specific antigen had been formed, for the faint globulin fuzz of the antigen antibody reaction was easier to pick up than a few sparsely separated ferritin granules.

Table 1. *Stages during transformation of Paramecium aurelia from antigenic type 168G to type 168D by growth at 35°, showing comparisons of the results by the three methods used for detecting antigen*

		Effect of antisera (dil. 1/50) on live organisms		Fluorescence following treatment with fluorescein-labelled anti-168D serum		Ferritin granules and globulin fuzz following treatment with ferritin-labelled anti-168D γ-globulin	
Stage	Time of growth at 35° (hr)	Anti-168G immobilization time (min.)	Anti-168D immobilization time (min.)	Pellicle	Cilia	Pellicle	Cilia
0	0	4	Unaffected	Nil	Nil	Nil	Nil
1	1–2	4	Unaffected	Nil	Nil	*Granules and fuzz at a few isolated sites	Granules and fuzz at a few isolated sites
2	3–5	4	R	*Faint green	Nil	Sites more numerous	Sites becoming more numerous
3	6	5	R	Green	Faint green	Sites very numerous	Sites becoming more numerous
4	7–8	*7	R	Green	Green	Granules and fuzz cover entire surface	Sites becoming more numerous
5	9	15	50	Green	Green	Granules and fuzz cover entire surface	Sites very numerous
6	18–20	Unaffected	4	Green	Green	Granules and fuzz cover entire surface	Granules and fuzz cover entire surface

R indicates retarded but not completely immobilized after 2 hr in serum; * indicates first definite detection of change of antigen by each method.

A preliminary experiment was done by treating paramecia in the process of transformation from 168G to 168D with ferritin-labelled antibody to the antigen 168G initially present. After growth for 9 hr at 35°, the results indicated that antigen 168G was still distributed fairly homogeneously over the surface of the cilia, but was present only at small isolated sites on the pellicle. At this stage the immobilization times of live organisms were much the same in antisera to each of the antigens, indicating that a fair amount of each antigen was present on the cilia.

Attempts to identify intracellular antigens

Since the ferritin labelling and counterstaining proved to be a specific and sensitive technique it was hoped to use it to examine the interior of the paramecia for the presence of immobilizing antigens. It has been shown by earlier experiments

with whole paramecia, by using the fluorescence technique (Beale & Kacser, 1957), that antibody does not penetrate the cell-wall. All attempts to label Araldite sections of paramecia with ferritin-labelled antibody failed, ferritin being deposited over the entire surface, as found with methacrylate sections by Baxandall, Perlmann & Afzelius (1962). Embedding the paramecia in the water-soluble polyepoxide Durcupan (X133/2097), according to Staubli (1960) and Leduc & Bernhard (1962), was also unsuccessful; again the ferritin was deposited non-specifically on the sections, as with Araldite.

DISCUSSION

The fine structure of the paramecium was found to be very well preserved in Araldite for electron microscope studies, especially the outer membranes, as previously noted by Stewart & Muir (1963), since there was little polymerization damage on hardening (Glauert & Glauert, 1958). Counterstaining of the sections with uranyl acetate + potassium permanganate was necessary, however, to bring out the fine detail of the membranes etc., because of the low contrast of embedded material in Araldite. The nature of the membranes and the general ultrastructure of the paramecium, as well as the position of the ferritin- and globulin-labelling of the antigen on the membranes, are of importance in the discussion of the possible nature of the molecular transfer in the formation of the immobilization antigen.

The following details of the general ultrastructure of the paramecium were noted. The ciliary 'corpuscle', in the terminology of Ehret & Powers (1959), consists of the cilium, arising from the kinetosome, from the centre of the pit of the circumciliary space, the walls of which enclose the peribasal space (Pl. 2, fig. 3). The parasomal sac opens into the circumciliary space (Pl. 2, fig. 4). These ciliary corpuscles alternate with the trichocysts, and the various structures under the electron microscope make up the characteristic silver-line system of light microscopy (Pitelka, 1963). The cross-sections of the cilia show the usual arrangements of nine double peripheral fibrils and two single central fibrils, with the nine secondary fibrils sometimes also visible.

The outer membranes of the paramecium consisted of two distinct membranes as previously noted (Stewart & Muir, 1963; Mott, 1963), the outermost pellicular membrane being continuous over the entire surface of pellicle and cilia, and the inner peribasal membrane forming a continuous lining around the peribasal space (Pl. 2, fig. 3). Over the peribasal space these two membranes were separated by varying distances, the two together generally making a double membrane about 250 Å wide. Both these membranes were seen to consist of the classic unit membrane, the '3-ply' sheet (Robertson, 1960) about 75 Å wide made up of two dark-staining outer layers each about 25 Å thick, separated by a clear layer about 25 Å wide, these membranes being considered to be a bimolecular leaflet of lipid covered on both sides by layers of protein (Stoeckenius, 1962). No pores were seen in these membranes, but there was frequent overlap of the membrane layers. Below the peribasal membrane, a dark-staining thick homogeneous layer or membrane was seen (Pl. 2, fig. 3), about 100 Å wide, and noted previously by Pitelka (1961) in Tetrahymena; this appeared to be continuous with the septum across the base of the cilia.

The studies with ferritin labelling presented here confirm previous indications that the immobilization antigen is situated on the surface of the pellicle and cilia of the

paramecium. From the transformation experiment, it was found that antigen appeared initially at isolated sites on pellicle and proximal ends of cilia, the number of sites gradually increasing until the antigen completely covered first the pellicle and later the cilia. Comparison of the three concurrent tests showed that the ferritin-labelling technique was much more sensitive than the fluorescent-labelling technique, both being more sensitive than the immobilization test which only reveals ciliary antigen and then only when present in fairly large amount. Stages of transformation with ferritin labelling were examined to see whether the ferritin granules, indicating 'new' antigen formation, bore any relationship to particular surface structures. No evidence was found of flow of antigen along the surface from any particular point of emergence, such as possible pores in the membranes. No pores were seen, though frequently a folding was noted (Pl. 2, fig. 3); however, the appearance of ferritin granules did not particularly coincide with any of these folds. Ferritin granules, in the transformation studies, were not seen to be particularly associated with the openings of the parasomal sac, when seen in several sections, nor in the area of the sac generally, nor did there appear to be any flow of antigen from this area. It was noticed on examining the micrographs that the density of individual ferritin granules and their distance from the pellicular membrane varied greatly. This was also noted in the micrographs of ferritin labelling by Morgan, Rifkind & Rose (1962). Both these observations could be due to the thickness of the sections, and the fact that the membrane was not perpendicular to the plane of section, particularly when there was a protuberance of the surface, as often occurs on cilia (Pl. 1, fig. 1). Also, the ferritin granules were seldom very close to the membranes; this was possibly due to the fact that the ferritin label might be situated on the globulin at a distance from the active site which combined with the antigen. The varying distances might then be due to the antibody molecule either being attached to antigen by both terminal active sites, or opening out and attaching to antigen by only one end (Lafferty & Oertelis, 1963; Feinstein & Rowe, 1965).

At the degree of sensitivity of the fluorescent labelling method it was shown previously (Beale & Mott, 1962) that the immobilization antigen was not present in the interior cytoplasm of the organism. However, this method is not so sensitive as ferritin labelling so that it is possible that extremely small amounts of antigen in the cytoplasm might be undetected by the fluorescence technique. The presence of a small number of antigen molecules in the internal cytoplasm has therefore not been disproved and thus the immobilization antigen might be formed either in the internal cytoplasm or on the surface membranes. If the antigen is formed in the cytoplasm, antigen molecules would then have to pass to the exterior of the paramecium. The purified soluble immobilization antigens have been studied in some detail and have been subjected to various immunological, chromatographic and electrophoretic tests. The antigens are proteins of molecular weight about 250,000 (Preer, 1959; Bishop, 1961). It would appear that the immobilization antigens might be synthesized on the ribosomes in the cytoplasm, where they could exist as formed antigen molecules, not necessarily very many at any one time, and as such pass out of the cell. These ribosomes might be associated with the membranes of the endoplasmic reticulum (Palade, 1956) which are known to be sites of active synthesis. Ribosomes are also present on the cytoplasmic side of external cell membranes and the antigen could thus be formed close to the cell surface. If the antigen itself is formed on the

ribosomes, internal ferritin labelling might show antigen associated with polyribosomes made up of a definite number of ribosomes, since various classes of polysomes appear to synthesize similar polypeptide chains as shown in specialized cells such as reticulocytes, making a single protein (Marks, Burka, Rifkind & Danon, 1963; Rich, Warner & Goodman, 1963). The alternative to the antigen being synthesized within the cell and moving outside it, is that the information only, as messenger RNA, passes from the gene to the external membranes and the antigen is synthesized there. There is the possibility that this RNA could take the form of an intermediate particle located in the cytoplasm, similar to the metagons in the killer system in paramecium (Gibson & Beale, 1963). The fibrils, both in the cilia and in the cytoplasmic network, are also known to have enzymic activity (Nelson, 1959); Pitelka (1963) suggested they might transmit information or messenger substances. These systems would necessitate either the antigen protein or the RNA, both molecules of molecular weight about 200,000, moving to the exterior of the cell. From the study of the ultrastructure, it appears this passage might be possible, not through openings such as pores or the parasomal sac, but through the membranes themselves. If the membranes were static structural entities, there would seem to be several mechanical barriers to a large molecule passing to the exterior. Three '3-ply' membranes and the large peribasal space separate most of the cytoplasm from the exterior, as well as the dense homogeneous layer or membrane mentioned earlier. This dense layer also appears to be continuous with the septum across the base of the cilium, which would appear to be a barrier from the general cytoplasm to the interior of the cilia. It is well known, however, that membranes contain enzymes and that there is a flow of molecules through cell surfaces in metabolism. It is thought that the cell membranes are impermeable to a great many substances unless metabolized by the enzymes in and behind the membranes (Mitchell & Moyle, 1959). This membrane-selective chemical mechanism of Davson & Danielli (1952) might account for the passage of large molecules, such as antigen or RNA, through the membranes to the exterior, whereas other molecules, such as the globulin of the antibody (of molecular weight 156,000) were unable to penetrate the cell-wall, possibly because of the lack of suitable enzymes in the membranes. Mechanical transport through pores in the membranes would not give this degree of specificity. It is possible that these enzymes take part in the transport of special molecules by the formation and dissolution of the membranes which are in a constant state of flux and transformation. It has been suggested for the cytomembranes (Novikoff, Essret, Goldfischer & Heus, 1962) that they have functional rather than structural continuity, although they are thought to be more or less continuous with the nuclear membrane and possibly also the cell surface (Palade, 1956; Gray, 1964), the surface also being capable of active transformation and vesiculation (Bennett, 1956). Direct observation of the antigen molecules on the membranes is suggested by the fact that these molecules are probably present in large numbers, as indicated by the thickness of the globulin fuzz and by the simple calculation of the probable number of molecules per unit area of surface. It would appear from the present study that the actual antigen molecules cannot be demonstrated on the membranes with the techniques and magnifications used. The study of the membranes by other techniques and at higher magnifications, in addition to the application of the ferritin-labelling technique to the interior of the organism, may be of value in finding possible sites of synthesis of the immobilization antigens.

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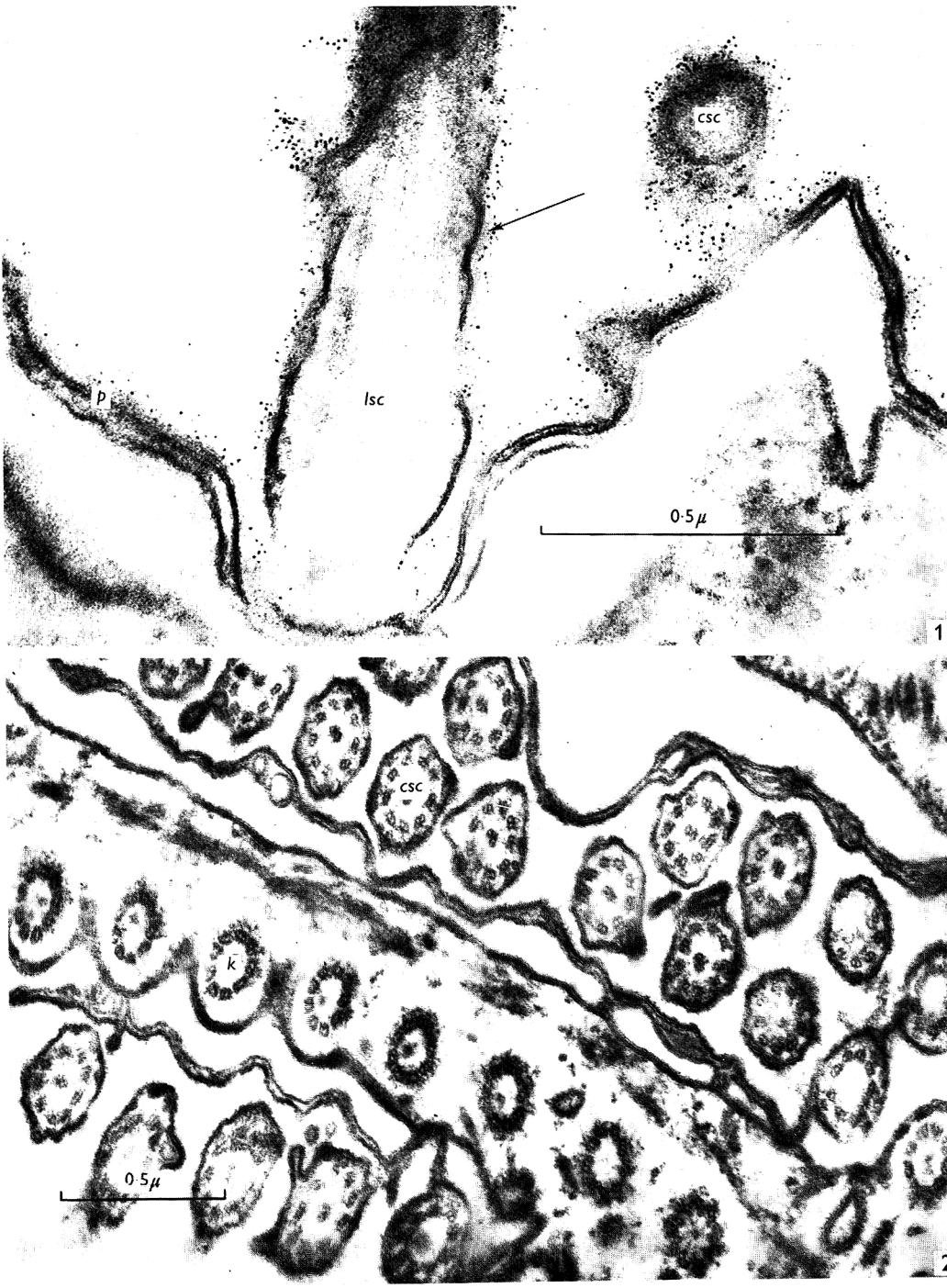

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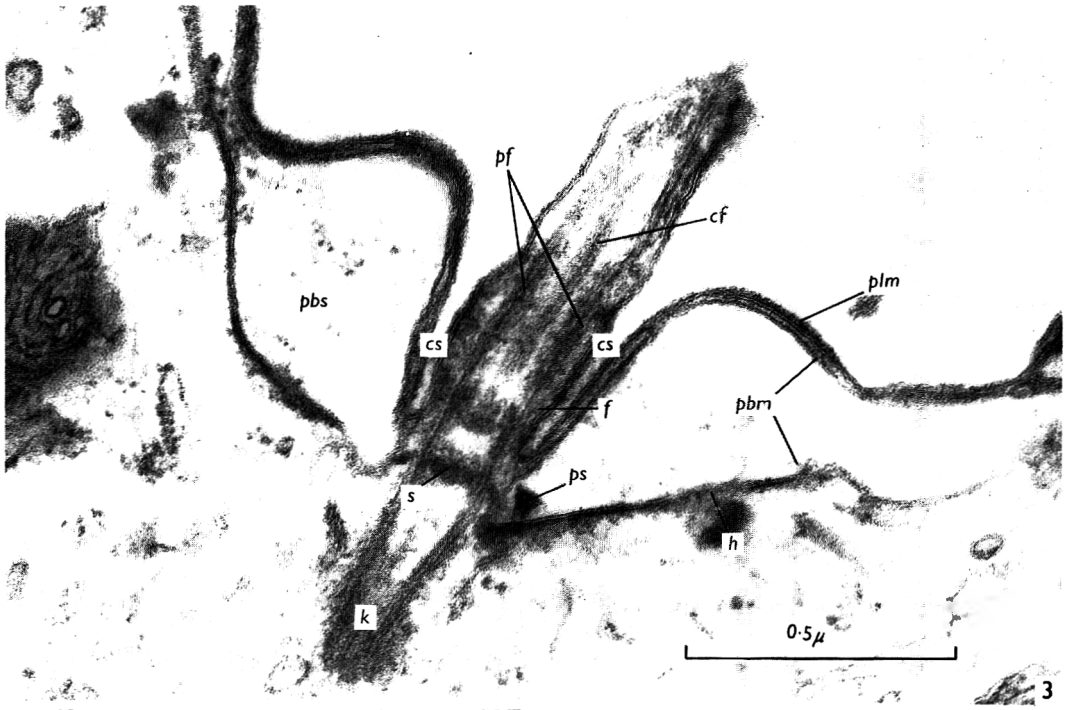
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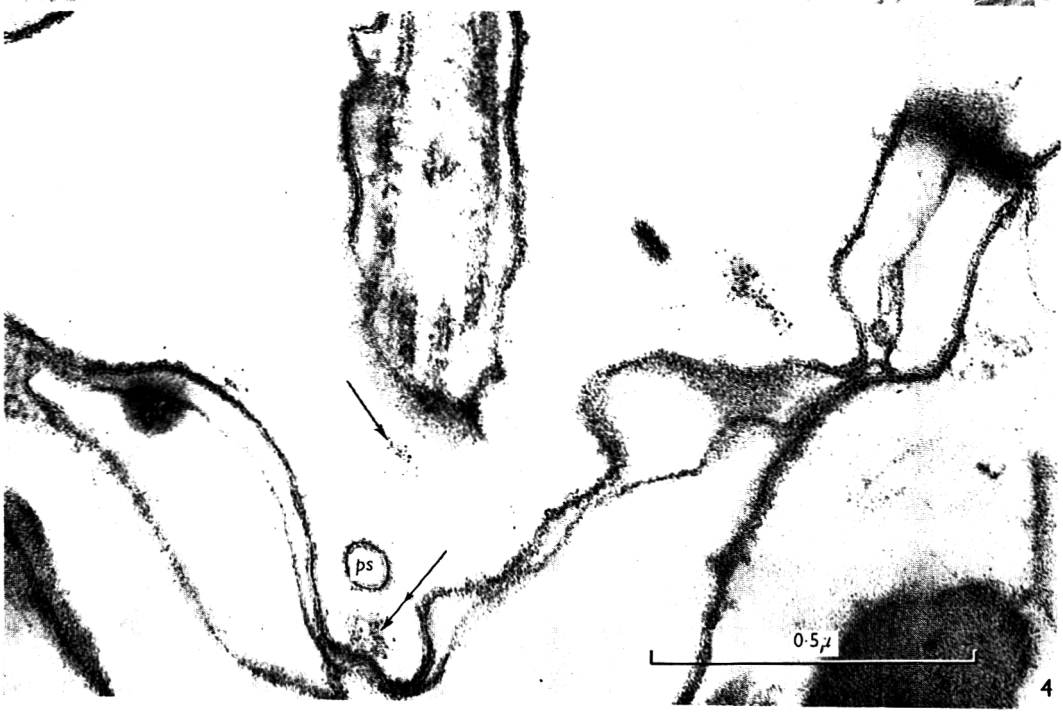
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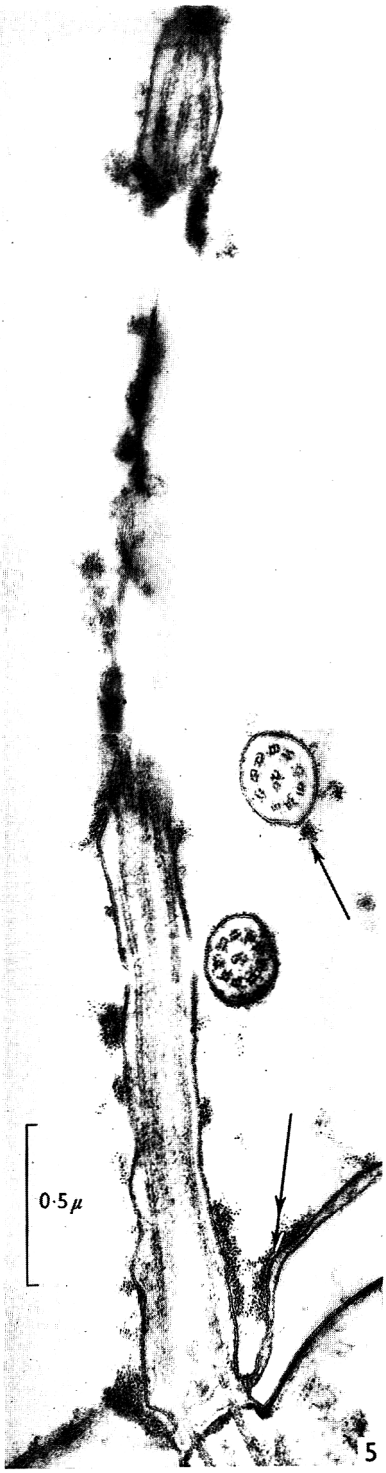
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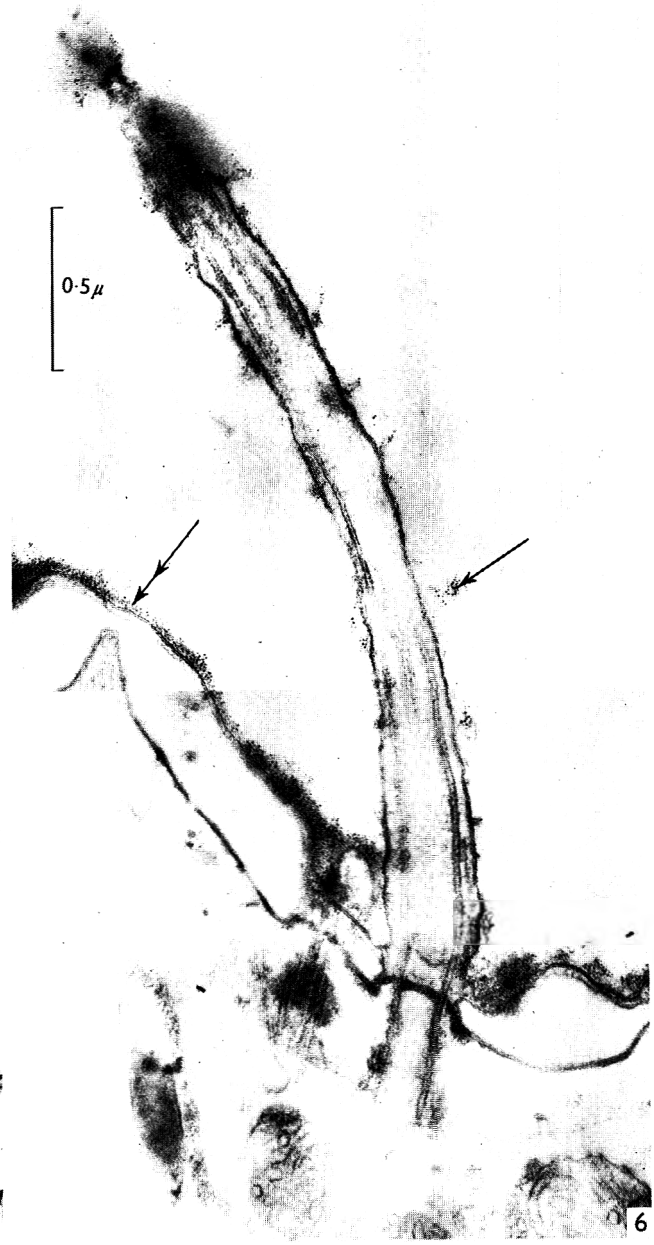
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EXPLANATION OF PLATES

Key to plates. *cf* = central fibrils; *cs* = circumciliary space; *csc* = cross-section of cilium; *f* = fold; *h* = homogeneous layer; *k* = kinetosome; *lsc* = longitudinal section of cilium; *p* = pellicle; *pbm* = peribasal membrane; *pbs* = peribasal space; *pf* = peripheral fibrils; *plm* = pellicular membrane; *ps* = portion of parasomal sac; *s* = septum.

Plate 1

Fig. 1. Unstained section of cilium of a Paramecium fixed and treated with ferritin-labelled homologous antibody, showing ferritin granules (arrow) on pellicle and cilium. Note poor structural detail in this unstained section.

Fig. 2. Counterstained section of gullet area of an homologous paramecium fixed and treated with ferritin-labelled antibody, showing the usual '3-ply' membranes and no globulin fuzz or ferritin granules in this area. Cross-sections of cilia and kinetosomes are seen.

Plates 2 and 3

Comparisons of counterstained sections of fixed paramecia treated with ferritin-labelled antibody to the 'new' antigen being formed during antigenic transformation.

Plate 2

Fig. 3. At the beginning of the transformation experiment, after 'nil hours' growth at 35°. No fuzz or ferritin granules are present on the surface of either pellicle or cilium. Note single '3-ply' membrane around the cilium, and the double '3-ply' membrane over the pellicle. (Reproduced by kind permission of the Royal Microscopical Society from the report of the proceedings of the Symposium on Cytochemical Progress in Electron Microscopy, Oxford, 1962. Published in the *J. R. Micr. Soc.* **81**, 1963.)

Fig. 4. After 1 hr of growth at 35°, showing very few isolated sites of ferritin granules and faint globulin fuzz on pellicle (double arrow) and cilia (arrow). The possible opening of the parasomal sac is seen below the cilium.

Plate 3

Fig. 5. After growth for 6 hr at 35°. The globulin fuzz and ferritin granules can be seen at a large number of separate sites on the pellicle (double arrow) and also on the longitudinal and cross-sections of the cilia (arrow).

Fig. 6. After growth for 7 hr at 35°. The globulin fuzz and ferritin granules are seen to cover the entire pellicle (double arrow), but are still in isolated sites on the cilium (arrow).

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A Note on the Taxonomic Status of Strains Like 'Campo,' Hitherto Classified as *Mycoplasma hominis*, Type 2

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SUMMARY

The classification of strains similar to Mycoplasma strain PG 27 of 'Campo' as *Mycoplasma hominis* Type 2 should be withdrawn. These strains have now been identified as *M. arthritidis*.

Nicol & Edward (1953) in an examination of 91 strains of Mycoplasma, isolated by them from the human genital tract, showed that all except one belonged in a single species, then termed 'Human Type 1'. An additional 13 genital strains were received from other laboratories. Of these, 9 from three laboratories were found to belong to 'Human Type 1'. A tenth strain was identified as a different species, later to be called *Mycoplasma fermentans*. The other three strains differed serologically from the 'Type 1' and were provisionally classified as 'Human Type 2'. Edward (1954) noted later that the 'Human Type 2' strains produced abscesses in mice when inoculated subcutaneously together with agar. This was a property that 'Human Type 1' strains did not have, but was one shared by certain rat isolates. On the other hand, Ruiter & Wentholt (1952) described abscess formation when their 'G' strain (*M. fermentans*) was inoculated into the foot-pads of mice. Unfortunately, no serological comparison was made at the time between the 'Human Type 2' and the rat strains. Edward & Freundt (1956) suggested a classification and nomenclature for the Pleuropneumonia Group (family Mycoplasmataceae), in which the 'Human Type 1' and 'Human Type 2' strains were regarded as two varieties of a single species, namely *M. hominis* Type 1 and *M. hominis* Type 2, the representative strain of the latter being strain 'Campo' (PG 27), maintained at the Wellcome Research Laboratories. Classification was proposed in this way because the two groups of strains were similar in their biological and cultural properties and apparently had a common source; they differed only serologically. Lemcke (1964) pointed out that strain 'Campo' was indistinguishable serologically, using the complement fixation test, from five rat strains of *M. arthritidis*. She therefore suggested that strain 'Campo' should no longer be regarded as a distinct species. *M. arthritidis* has been frequently found in rats and was a recognized pathogen for that animal; its natural host seemed to be the rat and thus 'Type 2' strains in man should be regarded as commensals or saprophytic contaminants. Use of the agar-gel double diffusion technique revealed a slight difference between 'Type 2' strains and *M. arthritidis*, but not sufficient to justify regarding the former as a distinct species (Lemcke, 1965). In confirmation, Leach (1965, per. comm.) by using the agglutination reaction obtained identical titres in reciprocal tests with both strain

'Campo' (PG 27) and *M. arthritidis* antigens against rabbit antisera to these antigens. In the growth-inhibition test, antisera to strain 'Campo' and to *M. arthritidis* inhibited both strains. In these tests the designated representative strain of *M. arthritidis*, namely strain 'Preston', was used. A re-examination of biological properties failed to reveal any difference between strain 'Campo' and strain 'Preston'.

The evidence is, therefore, that the strains examined in England and originally classified as a Type 2 variety of *Mycoplasma hominis* are strains of *M. arthritidis*, an already established species of Mycoplasmataceae. (Sabin, 1941) *M. arthritidis* is a well-known pathogen of rats and there seems no justification for including *M. hominis* Type 2 among the 'human' species of *Mycoplasma*, although many biochemical studies have been done with 'Campo' and related strains as representative of human Mycoplasma (see Hayflick & Chanock, 1965).

There remains the question of the real origin of strains, alleged to have been isolated originally from the human genital tract and now, after much subcultivation, found to be identical with *Mycoplasma arthritidis*. These strains are few. Strain 'Campo' was isolated from a human male urethra about 1939–40. Two lines of this strain were examined by Nicol & Edward (1953): one line was from the laboratory of origin and the other from another laboratory; both behaved similarly. The two other strains with similar serological properties were strain 07 and strain 39. In addition to these strains, which on examination have been shown to belong to *M. arthritidis*, Nicol & Edward (1953) referred to reports of the isolation of ten other strains, isolated from human genital tracts and found to be serologically similar to strain 'Campo'. Bailey *et al.* (1961) also reported two other strains as serologically similar to their strain of 'Campo', one (CH) isolated by them from the human genital tract and the other (sp-1) isolated from a HeLa cell line.

One possible explanation of the 'Campo'-like strains is that they have resulted from laboratory misadventures occurring during their long subcultivation, allowing the replacement of a human strain by a rat strain. Many of the risks encountered in subcultivation are obvious. Moreover, since most species of Mycoplasma do not differ from each other significantly in their colonial appearances, a contaminating Mycoplasma may be unrecognized and eventually outgrow and replace the original strain. Edward (1950) described the properties of a strain he believed he had isolated from mice, only to discover later (Edward, 1954) that it was identical with the human genital strain he had been maintaining. Freundt (1954) claimed to have made two isolations of *Mycoplasma mycoides* var. *mycoides* from the human genital tract, but these were made at a time when a bovine strain of this species was under study in his laboratory. He noted that the fermentation of mannose was slower with the genital strains than with the bovine strain. Nevertheless the most likely explanation of his genital isolations is that they resulted from laboratory pick-ups. It is perhaps significant that *M. mycoides* has never been isolated from man subsequently, although very many strains have been carefully examined. Lemcke & Csonka (1962) reported that a strain, isolated by them from the external genitalia of a man, had been identified serologically as a mouse strain. A subsequent re-examination of this strain by Dr R. M. Lemcke (personal communication) showed that it was a strain of *M. hominis* (type 1), the original erroneous identification having been made because a mouse strain was under investigation in that laboratory at the same time.

If this theory of contamination is correct, there was, and may still be, a strain of

'Campo' representative of the original genital isolate. This must be borne in mind in considering reports that genital isolates have been found serologically similar to strain 'Campo'. The identity of these isolates and whether they were different from the common human genital species, *Mycoplasma hominis* (type 1), must remain uncertain.

Obviously the alternative hypothesis is that there have in fact been several isolations of *Mycoplasma arthritidis* in the U.S.A. from the human genital tract. Since *Mycoplasma* spp. in general show a high degree of host specificity it would be important to know whether a pathogen of rats could also be commensal or a pathogen for man; further work is highly desirable. The need for a reference laboratory or laboratories is becoming more and more apparent in this now rapidly opening field, to examine isolates of human and animal origin. There is also a need, not only for extending, but also for standardizing the biological tests, such as the determination of haemolysis in blood agar. The differences between strain 'Preston' and strain 'Campo' originally noted by Edward (1954) were not confirmed in a re-examination by Dr R. H. Leach (personal communication). The observations of Somerson, Taylor-Robinson & Chanock (1963) emphasize the difficulties with this test of haemolysis.

The conclusion must be that the classification of strains similar to strain PG 27 of 'Campo' as *Mycoplasma hominis* Type 2 should be withdrawn, since these strains have now been identified as *M. arthritidis*.

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The Influence of Temperature and pH Value on the Macromolecular Composition of Magnesium-limited and Glycerol-limited *Aerobacter aerogenes* Growing in a Chemostat

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SUMMARY

Progressive alteration of the incubation temperature of *Aerobacter aerogenes* cultures, growing in a chemostat at a fixed dilution rate, caused a progressive change in bacterial concentration and in bacterial RNA, carbohydrate and protein contents; the DNA content did not vary significantly. The change in bacterial RNA concentration was associated with a variation in bacterial ribosome content, similar to that found in organisms grown at a fixed temperature but different dilution rates. Changes in bacterial yield also resulted from alterations in the pH value of the culture, but corresponding changes in bacterial RNA, carbohydrate and protein contents were small.

INTRODUCTION

A close correlation has been found between the RNA content of organisms and the rate at which they grow (Wade & Morgan, 1957; Neidhardt & Magasanik, 1960; Dean, 1962; Rosset, Monier & Julien, 1964; Sykes & Tempest, 1965). As these variations in cellular RNA content appeared to be due largely, if not entirely, to changes in the ribosome content of the organisms (Ecker & Schaechter, 1963*a*; Kjeldgaard & Kurland, 1963), and as ribosomes seemed to be the primary sites of protein synthesis, it was suggested that in growing organisms the rate of protein synthesis per ribosome particle was constant and independent of growth rate (Schaechter, Maaløe & Kjeldgaard, 1958; Ecker & Schaechter, 1963*b*). Apparently opposed to these findings was the further observation of Schaechter *et al.* (1958) that the macromolecular composition of *Salmonella typhimurium* organisms was not a function of growth rate when this was altered by changing the temperature of cultivation. This latter investigation was done with organisms grown under conditions of unrestricted growth, in a batch culture. In the chemostat the growth rate is controlled and it is possible, therefore, to vary the temperature of cultivation without simultaneously altering the growth rate of organisms in the culture.

The present paper reports the changes in RNA, DNA, carbohydrate and protein contents of *Aerobacter aerogenes* which occurred when the incubation temperature of the cultures, growing at a constant dilution rate, was progressively altered. These changes are shown to be similar to those reported by Tempest, Hunter & Sykes (1965) to occur in cultures of this organism when the dilution rate was progressively changed but the growth temperature maintained constant. By comparison, changes

in the culture pH value are shown to have only a small influence on the gross macromolecular composition of the organisms. These results, and some of those of Schaechter *et al.* (1958), are interpreted in terms of environmental effects on ribosome synthesis and activity. A preliminary report on our findings has been published (Tempest & Hunter, 1965).

METHODS

Organism. Aerobacter aerogenes (NCTC 418) maintained by monthly subculture on tryptic meat digest agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0·5 l. chemostats of the design described by Herbert, Phipps & Tempest (1965). The temperature differential at each set point was less than 0·2° and the pH differential less than 0·2 unit. Fluctuations in dilution rate, due to changes in culture volume and medium flow rate, were always less than $\pm 5\%$ of the desired value.

The composition of the media, and other conditions, were the same as those used by Tempest *et al.* (1965).

Viability measurements. These were made with the slide-culture technique of Postgate, Crumpton & Hunter (1961).

Fractionation procedure. 1–2 l. volumes of effluent culture were collected overnight in ice-cooled receivers, transferred to 250 ml. centrifuge buckets and sedimented (16,000*g*, 20 min.) in an MSE High Speed 17 refrigerated centrifuge. Packed bacteria were resuspended in ice-cold buffer (0·01 M-tris + 0·001 M-magnesium acetate; pH 7·5) and again sedimented (25,000*g*, 20 min.). Bacterial pastes were transferred to a Hughes press (Hughes, 1951), pre-cooled to −20°, and the organisms crushed. The crushed pastes were diluted with buffer (4 ml./g. frozen crushed paste, plus a crystal of deoxyribonuclease) and homogenized. Homogenates were centrifuged (20,000 rev./min., 45 min.) in a no. 30 rotor of a Spinco model L ultracentrifuge and the clear supernatant fluids (labelled 'Crude extract') were collected. These fluids were again centrifuged (40,000 rev./min., 3½ hr.) in a no. 40 rotor of a Spinco model L ultracentrifuge and separated into two fractions—a second supernatant fluid (labelled 'Supernatant'), and a pellet fraction (labelled 'Ribosomes') which was resuspended in ice-cold buffer to a convenient volume (10 ml. for material from 10 g. crushed paste). The RNA and protein contents of all samples were determined and the values obtained adjusted to correspond with a protein value of 1·000 in the 'Crude extract'.

Analytical procedures. These were identical with those used previously by Tempest *et al.* (1965).

RESULTS

Effect of temperature on the steady-state macromolecular composition of organisms growing at a fixed dilution rate

There is a maximum dilution rate beyond which cultures of any organism cannot be maintained in the chemostat. This 'critical' value (D_c) corresponds with the maximum specific growth rate of that organism (μ_m) in the environment provided (Herbert, Elsworth & Telling, 1956); it varies with incubation temperature, pH value and chemical complexity of the medium. In a simple salts medium, regulated at pH 6·5 and with glycerol as the carbon source, cultures of *Aerobacter aerogenes* have a minimum doubling time of 50 min. at 35°; at 25° this time is increased to about

90 min. Therefore in order to maintain the growth rate of this organism at a constant value whilst varying the incubation temperature it was necessary to operate the chemostat at a dilution rate substantially less than the 'critical' value for cultures growing at the least favourable temperature. As the value of D_c, at 25°, was approximately 0·45 vol./hr., we chose a dilution rate of 0·2 vol./hr. for our experiments. We progressively altered the temperature of cultivation from 40° to 25°, in increments of 5°, and determined the effect of these changes on the steady-state composition of organisms. Both glycerol-limited and Mg^{2+}-limited cultures were investigated.

Table 1. *Macromolecular composition of Aerobacter aerogenes grown at a fixed dilution rate and at different temperatures*

All the analytical data contained in this table are average values obtained from at least two samples (harvested and processed on different days) grown at each steady-state temperature.

		% of dry weight				mg./ml. culture				
Temp. (° C.)	Dilution rate	RNA	DNA	Carbohydrate	Protein	Dry wt. organisms	RNA	DNA	Carbohydrate	Protein
				Glycerol-limited growth						
40	0·20	8·6	2·4	2·9	74·1	4·10	0·35	0·10	0·12	3·04
35	0·20	10·1	3·3	3·3	68·4	4·86	0·49	0·16	0·16	3·32
30	0·21	11·0	2·6	3·6	67·5	4·71	0·52	0·12	0·17	3·18
25	0·22	11·8	2·7	8·9	59·4	4·91	0·58	0·13	0·43	2·91
				Mg^{2+}-limited growth						
40	0·18	9·4	2·9	2·1	69·0	4·14	0·39	0·12	0·09	2·86
35	0·18	10·7	2·9	2·2	70·0	4·12	0·44	0·12	0·09	2·88
30	0·19	12·4	2·9	2·6	67·5	3·46	0·43	0·10	0·09	2·33
25	0·19	14·9	2·8	4·3	68·9	2·82	0·42	0·08	0·12	1·94

The results obtained are shown in Table 1 and clearly differ from those reported by Schaechter *et al.* (1958) in that changing the temperature of cultivation produced marked changes in the RNA and carbohydrate content of the organisms. Similar changes were evident in both types of culture, indicating that the changes were independent of the nature of the growth-limiting component of the medium. Furthermore the stoichiometry between Mg^{2+} and RNA, previously observed in Mg^{2+}-limited cultures of this organism (Tempest *et al.* 1965), was also apparent here; the culture-RNA content varied only slightly with growth temperature and changes in cellular RNA content were matched by gross changes in the steady-state concentration of organisms in the culture. In contrast, in the glycerol-limited culture both the yield of organisms and their RNA content increased with decreasing growth temperature; the culture-RNA value increased by 66% as the incubation temperature was lowered from 40° to 25°.

The above pattern of changes in macromolecular composition and yield of organisms was very similar to that reported to follow changes in growth rate of organisms at a fixed temperature (Herbert, 1961; Neidhardt, 1963; Tempest *et al.* 1965); this was particularly true for the changes in cellular RNA content. Therefore we determined the distribution of RNA and protein in fractions from organisms grown at two different temperatures in order to establish whether these changes reflected

changes in the ribosome content of organisms similar to those reported by Ecker & Schaechter (1963*b*) to follow changes in growth rate. The data obtained are shown in Table 2, which shows that with glycerol-limited and with Mg^{2+}-limited organisms a decrease of 10° in the growth temperature produced a marked increase (about 30 %) in their ribosome contents. The amounts of RNA and protein which could be sedimented under the prescribed conditions increased, but not in an identical manner. Thus the RNA:protein ratios in the 'Ribosome' fractions changed (from 0·37 to 0·57 in the material from Mg^{2+}-limited organisms, and to a lesser extent in the material from glycerol-limited organisms). The change in composition of this fraction is similar to that observed by Tempest *et al.* (1965) in fractions from organisms grown at a fixed temperature but at different dilution rates.

Table 2. *Distribution of RNA and protein in fractions from extracts of glycerol-limited and Mg^{2+}-limited Aerobacter aerogenes organisms grown at different temperatures*

	35°		25°	
	Protein	RNA	Protein	RNA
	Glycerol-limited growth			
Crude extract	1·000	0·194	1·000	0·236
Supernatant fluid	0·685	0·044	0·585	0·040
Ribosomes	0·317	0·141	0·398	0·187
Ribosomal RNA + protein	0·458		0·585	
	Mg^{2+}-limited growth			
Crude extract	1·000	0·175	1·000	0·262
Supernatant fluid	0·686	0·048	0·621	0·050
Ribosomes	0·318	0·118	0·369	0·211
Ribosomal RNA + protein	0·436		0·580	

Effect of pH value on the steady-state macromolecular composition of organisms growing at a fixed dilution rate

Although organisms are not able to maintain a temperature differential between themselves and their environment they may well maintain a pH differential; alteration of the culture pH value may not, therefore, effect a corresponding change in intracellular pH value. The fact that relatively small changes in culture pH value influence the enzymic constitution and content of organisms (Gale & Epps, 1942) suggests, however, that some change in intracellular pH value does follow a gross change in the environmental pH value. The optimum pH value for growth of *Aerobacter aerogenes* occurred within the range pH 6–7, though growth was not totally inhibited at values as low as pH 4·5 or as high as pH 8·5. The effect of progressively altering the culture steady-state pH value from 4·5 to 8·5, in steps of 1 pH unit, on the composition of organisms in glycerol-limited and Mg^{2+}-limited cultures (temperature, 35°; dilution rate, 0·2 vol./hr.) is shown in Table 3. It is apparent here that the changes in macromolecular composition of organisms with pH value were much less than those observed to follow changes in the temperature of cultivation. The main effect of changing the pH value, in both types of culture, was on the bacterial yield, which was maximal at the optimum pH value for growth of this organism. In the Mg^{2+}-limited culture the RNA:Mg^{2+} ratio remained constant, though some change

Table 3. *Macromolecular composition of Aerobacter aerogenes organisms grown at different pH values (temperature and dilution rate constant)*

All the analytical data contained in this table are average values obtained from at least two samples (harvested and processed on different days) grown at each steady-state pH value.

		% of Dry weight			mg./ml. culture			
pH value	Dilution rate	RNA	Carbohydrate	Protein	Dry wt.	RNA	Carbohydrate	Protein
			Glycerol-limited growth					
4·5	0·18	10·8	4·0	73·0	4·27	0·46	0·17	3·12
5·5	0·19	11·1	4·2	71·5	4·31	0·48	0·18	3·08
6·5	0·18	10·5	3·3	71·0	4·58	0·48	0·15	3·25
7·5	0·19	10·3	3·1	73·1	4·24	0·43	0·13	3·10
8·5	0·19	10·3	3·2	74·3	3·31	0·34	0·11	2·46
			Mg^{2+}-limited growth					
4·5	0·18	11·7	4·2	68·1	3·60	0·42	0·15	2·45
5·5	0·18	11·3	2·9	69·5	3·80	0·43	0·11	2·64
6·5	0·19	10·3	2·3	68·1	4·14	0·43	0·10	2·82
7·5	0·19	10·6	2·3	73·7	3·87	0·41	0·09	2·85
8·5	0·21	12·8	2·5	67·5	3·19	0·41	0·08	2·15

in the RNA content of organisms occurred. However, there was little change in the culture-RNA content of glycerol-limited cultures with changes from pH 4·5 to 7·5. In both cultures there was a significant increase in the carbohydrate content of organisms when they were grown at low pH values.

DISCUSSION

Whereas varying the temperature of cultivation of *Salmonella typhimurium* in a batch culture has no effect on their gross composition (Schaechter *et al.* 1958) it clearly does so with chemostat cultures of *Aerobacter aerogenes* where the growth rate is controlled. Though superficially these observations appear to be contradictory they are, in fact, quite compatible assuming that the following two hypotheses are correct: (1) that the rate of protein synthesis per unit ribosome is constant and independent of growth rate (Schaechter *et al.* 1958); (2) that the concentration of ribosomes within an organism cannot exceed a certain fixed amount. One may assume the first hypothesis to be correct only for a fixed temperature and pH value. Lowering the environmental temperature would decrease ribosomal activity and the resulting decrease in protein synthesis would be reflected in a lower growth rate. If, however, growth rate is maintained at a constant value (as happens in the chemostat), then clearly the ribosome content of organisms must increase to offset the decreased ribosomal activity and to maintain the overall rate of protein synthesis constant. On the other hand, if the organisms already contain their maximum ribosome content (which one assumes to be so with fast-growing organisms in a batch culture) then a decrease in growth temperature cannot be compensated by a further increase in ribosome concentration, and the rate of protein synthesis must therefore decrease. Though growth rate is correspondingly decreased, the ribosome content remains maximal and thus the RNA content of organisms does not alter.

The above two hypotheses therefore satisfactorily account both for our observations and also those of Schaechter *et al.* (1958).

Assuming that organisms maintain a pH differential between their intracellular and extracellular environments, large variations in culture pH value may produce only small fluctuations (or none) of pH value within the bacteria. In Mg^{2+}-limited cultures, organisms grown at the optimum pH value do, however, have a lower RNA content than organisms grown at a higher or lower pH suggesting that a change in ribosomal activity occurs with alterations in culture pH value. In glycerol-limited cultures, on the other hand, the RNA content of organisms is virtually independent of culture pH value, indicating little if any change in their ribosome content, and therefore in their activity.

Changes in the concentration of bacteria in the Mg^{2+}-limited *Aerobacter aerogenes* culture with temperature could be correlated with changes in the RNA content of organisms and the maintenance of a stoichiometry between culture RNA and Mg^{2+} concentrations. With the glycerol-limited culture a decrease in growth temperature effected a marked increase in yield of bacteria, indicating a more efficient utilization of the carbon source. This increased efficiency probably resulted from a lower maintenance energy requirement (Marr, Nilson & Clark, 1963) for organisms growing at the lower temperatures. The culture pH value also influenced the bacterial yield which was maximal at the optimum pH value for rapid growth of *A. aerogenes*. Again the changes in bacterial concentration in the Mg^{2+}-limited culture paralleled changes in the RNA content of organisms. In the glycerol-limited culture the lower yields at the less favourable pH values probably resulted from an increased energy expenditure which would be required to maintain a large pH difference between the intracellular and extracellular environments.

It is of interest to note that both temperature and pH influenced the carbohydrate content of *Aerobacter aerogenes* which increased at the lower values. Even in cultures where the carbon + energy source was limiting growth, organisms grown at 25° contained substantially more carbohydrate than those cultured at 35° (8·9% versus 3·3% of the bacterial dry weight). Whether this extra carbohydrate was present as a storage compound (e.g. glycogen), or whether it resulted from an increase in structural (cell wall) polysaccharide material has not been determined.

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The Fine Structure of Sporangia of *Phytophthora erythroseptica* Pethyb.

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SUMMARY

Young non-germinating sporangia of *Phytophthora erythroseptica* contain nuclei and cytoplasmic organelles, similar in appearance to those in other fungi, and a conspicuous central and other vacuoles. The sporangial wall consists of an outer homogeneous layer and an inner vesicular layer, prominent in the apical region. Peripheral cytoplasmic vacuoles are frequent, particularly where the vesicular layer is well developed. In old sporangia, which lose the ability to germinate indirectly (i.e. to produce zoospores), there is no vesicular layer and there is a change in the nature of the storage material of the vacuoles. When young sporangia are stimulated to indirect germination by low-temperature treatment the central storage vacuole disappears and there is a marked elongation of mitochondrial cristae, consistent with an increased rate of respiration. The sporangial wall becomes three-layered with the development of an inner homogeneous layer which separates the plasma membrane from an increasingly prominent vesicular layer. In the region of the papilla the vesicular layer shows a striking increase in thickness; it is suggested that this layer and the inner homogeneous layer play an important part in zoospore discharge.

INTRODUCTION

Despite extensive studies of the morphology and physiology of sporangial formation and germination in Phytophthora and other fungi (review by Waterhouse, 1962) little is known of the fine structure of the sporangia of these organisms. Blondel & Turian (1960) described the ultrastructure of gametangia of Allomyces but the present authors are not aware of other studies of sporangial fine structure in fungi. As in other species of the genus *Phytophthora*, sporangia of *P. erythroseptica* may germinate either directly by germ tubes or indirectly by liberation of zoospores (Pethybridge, 1914; Blackwell, 1949). The age of a sporangium is an important factor in determining which of these two modes of germination will be adopted; old sporangia lose the ability to germinate indirectly (Blackwell, 1949). In an attempt to correlate these physiological changes with changes in small-scale morphology our description will include accounts of fine structure in ageing sporangia and in indirectly germinating sporangia, i.e. during zoospore formation.

METHODS

The organism examined in this study was *Phytophthora erythroseptica* Pethyb., the isolate used being one of those maintained in the collection of the Department of Cryptogamic Botany in the University of Manchester. The culture of *P. erythro-*

septica was incubated on pea broth (Leonian, 1934) at 22° for 7 days; the mycelial mats were then washed in sterile distilled water and transferred to Petri's solution to induce sporulation (Vujičić & Colhoun, 1966). Sporangia, together with small tufts of mycelium, were harvested from these mycelial mats 4 days after transfer (for young sporangia) or 8–10 days after transfer (for old sporangia). Specimens were fixed in 1% (w/v) osmium tetroxide in veronal+acetate buffer (pH 7·3; Palade, 1952) or in 2% (w/v) potassium permanganate in veronal+acetate buffer (pH 7·2; Luft, 1956). Fixation was carried out at 0–4° for 2 hr in OsO_4 or for 1 hr in $KMnO_4$.

After washing in tap water the specimens were dehydrated in ethanol and embedded in Araldite epoxy resin (Glauert & Glauert, 1958). The polymerized blocks were trimmed so that the face of each block contained one sporangium. Trimming of blocks was facilitated by special methods which will be described separately (Chapman, J. A. to be published 1966). Sections 600–800 Å in thickness were cut on a Huxley ultra-microtome with a glass knife. The sections were examined and photographed at magnifications ranging from ×1000 to ×20,000 in a Siemens Elmiskop I electron microscope operating at 80 kV and with double condenser illumination. Exposures were made on Ilford lantern contrasty plates.

The following specimens were examined: (*a*) young sporangia from 4-day cultures (10 sporangia examined); (*b*) old non-germinating sporangia from 8- to 10-day cultures (6 sporangia); (*c*) young sporangia stimulated by low-temperature treatment to germinate indirectly to produce zoospores (5 sporangia). In this treatment (*c*) pieces of sporulating mycelia, previously incubated at 22° for 4 days, were transferred to hanging-drop cultures and maintained at 13° for 1·5 hr and then brought to 22° for 10 min. before fixation. In control specimens, left unfixed, this treatment brought about zoospore discharge from 70 to 80% of the sporangia within 15 min. of raising the temperature (Vujičić & Colhoun, 1966). Although fixed specimens were all taken at the same time-point (10 min. after raising the temperature) they contained sporangia at various stages of indirect germination so that ultrastructural changes occurring during the advancement of indirect germination could be inferred.

RESULTS

The structure of the young non-germinating sporangium

The appearance of a typical sporangium of *Phytophthora erythroseptica* at a low electron optical magnification is shown in Pl. 1, fig. 1. A large vacuole, irregular in shape and with a maximum diameter of approximately 5 μ, occupies a central position and smaller vacuoles are abundant throughout the cytoplasm. Several nuclei can be seen. The sporangium is surrounded by an electron-transparent wall, and the plug connecting the sporangium to a hypha is shown.

Higher magnifications revealed that the structure of the nuclei, endoplasmic reticulum, mitochondria and other cytoplasmic inclusions resembled closely that described for other fungi (Blondel & Turian, 1960; Zalokar, 1961; Hawker & Abbott, 1963; Moore & McAlear, 1963*a*, *b*). The nuclei are relatively large bodies, 1·5–3 μ in diameter, roughly ellipsoidal in shape but with minor irregularities in outline. In permanganate-fixed material they possess a homogeneous finely granular appearance (Pl. 1, fig. 2). The nuclear envelope comprises a double membrane

traversed by numerous pores. Apparent continuity between the nuclear membrane and membranous organelles in the cytoplasm was occasionally noted.

Elongated cisternae of the endoplasmic reticulum occur widely in the cytoplasm (Pl. 1, fig. 2; Pl. 2, fig. 3). Ribosomal particles, free or attached to cisternal membranes, cannot be detected in permanganate-fixed material because of the failure of permanganate to preserve ribonucleoprotein (Luft, 1956). After osmium fixation ribosomes, occurring as electron-opaque particles 150 Å in diameter, are abundantly distributed throughout much of the sporangial cytoplasm (Pl. 3, fig. 4). The identification of these particles as ribosomes is supported by the work of Blondel & Turian (1960), who showed that similar particles were responsible for basophilia in hyphae and gametangia of the fungus Allomyces. These authors found that ribosomes were freely dispersed in the cytoplasm, and showed no preferential attachment to membranes of the endoplasmic reticulum; our results suggest that a similar situation exists in sporangia of *Phytophthora erythroseptica.*

Golgi dictyosomes were sometimes noted, usually in proximity to nuclei (Pl. 1, fig. 2). These took the characteristic form of stacks of close-packed flattened cisternae associated with smaller vesicles apparently pinched off from the cisternal margins; they closely resemble the Golgi dictyosomes found in higher plants and in other fungi (Moore & McAlear, 1963*a*). Similar Golgi dictyosomes, also adjacent to nuclei, were observed in older sporangia (Pl. 4, fig. 8).

Mitochondria of varied shape and size occur in abundance in the sporangial cytoplasm (Pl. 2, fig. 3). The inner of the two limiting mitochondrial membranes is infolded into the mitochondrial matrix to form the mitochondrial cristae. In young non-germinating sporangia these cristae are short and stumpy.

In addition to the large central vacuole (shown in part in Pl. 3, fig. 6), the young sporangium contains numbers of smaller storage vacuoles, also irregular in outline and with electron-transparent contents. Yamamoto & Tanino (1961) showed that sporangia of *Phytophthora infestans* were rich in glycogen and it is probable that this glycogen is located in the central and surrounding vacuoles; glycogen does not reduce osmium tetroxide nor (under normal conditions) permanganate (Revel, 1964) and the appearance of the vacuolar contents of *P. erythroseptica* (as in Pl. 3, fig. 6) is consistent with the presence of glycogen in the unstained areas (Zalokar, 1961).

Lipid inclusions occur in the form of small electron-transparent bodies of irregular shape with an intensely electron-opaque periphery (after osmium or permanganate fixation). The over-all diameter of these inclusions ranges from 0·3 to 0·8 μ (Pl. 3, fig. 4; Pl. 4, fig. 7). In osmium-fixed specimens some inclusions possess a fine lamellar structure with a periodicity of 60–70 Å (Pl. 3, fig. 4) suggesting that the contained material is phospholipid.

The peripheral regions of the sporangial cytoplasm contain rounded vacuoles about 0·3 μ in diameter and limited by a single membrane (Pl. 1, fig. 2; Pl. 2, fig. 3). These vacuoles, henceforth described as 'peripheral vacuoles', occasionally show an invagination of the limiting membrane penetrating almost to the centre of the vacuole (centre and bottom of Pl. 2, fig. 3). The finely granular contents possess an electron opacity comparable to that of the surrounding cytoplasm and in some instances the contents may be resolved as small vesicles up to 250 Å across (lower right of Pl. 2, fig. 3).

Particular interest attaches to the sporangial wall since this complex structure shows marked changes on ageing or during the course of indirect germination. The wall of a young sporangium (from a 4-day culture) consists of two layers outside the plasma membrane (Pl. 1, fig. 2; Pl. 2, fig. 3). The outer layer is structureless and (apart from a thin region bordering the sporangial surface) relatively electron transparent after both fixatives. It has a thickness of 2000–3000 Å. This layer would seem to be the one which gives a cellulose reaction when sporangia are treated with sulphuric acid and iodine in potassium iodide and examined by light microscopy (Pethybridge, 1914). The inner layer is variable in thickness and less electron transparent than the outer layer; its most characteristic feature is the presence of small vesicles, up to 500 Å in diameter and often occurring in groups. The vesiculated nature of this layer is particularly apparent in the wall surrounding the apical region of the sporangium (Pl. 2, fig. 3); in other regions the vesicular elements are more sparsely distributed (Pl. 1, fig. 2). In young sporangia a well-developed vesicular layer is associated with an abundance of peripheral vacuoles in the neighbouring cytoplasm (compare figs. 2 and 3) suggesting that these vacuoles may play an active part in vesicle formation. In such regions the plasma membrane is convoluted with numerous cytoplasmic extensions protruding into the vesicular layer (Pl. 2, fig. 3).

Under the light microscope the papilla, which is involved in the liberation of zoospores in indirect germination, appears as a mucilaginous thickening of the apical wall (Blackwell, 1949). In young non-germinating sporangia the papilla is inconspicuous and this may account for our failure to observe the papillar region in ultra-thin sections of such sporangia. Sections of papillae in older sporangia and in sporangia stimulated to indirect germination were readily obtained.

The plug connecting the hypha to the base of the sporangium serves to separate the hyphal contents from those of the sporangium. It consists of a greatly thickened region of wall interspersed with aggregates of electron-opaque material (Pl. 3, fig. 5). A distinct boundary exists, however, between the outer wall and the plug material. The appearance of this material suggests that the plug is formed in layers, possibly by intussusception of new layers between old ones. During its formation the plug always bulges into the sporangium but it may also bulge to a lesser extent into the hypha.

The structure of old non-germinating sporangia

The most conspicuous cytoplasmic change taking place on ageing occurs in the central vacuole, in which the contained material becomes uniformly electron transparent (Pl. 4, fig. 7). This is consistent with the light optical evidence that the storage material of the sporangium is transformed into some other reserve material (Blackwell, 1949). Lipid inclusions tend to be more numerous in old sporangia.

The sporangial wall consists of a single layer only (Pl. 4, fig. 8). The vesicular layer was absent and the outer structureless electron-transparent layer, now decreased in thickness to 1500–2000 Å, is directly apposed to the plasma membrane.

The papilla in an old sporangium appears as a homogeneous layer, finely granular after osmium fixation; it abuts directly on to the plasma membrane (Pl. 4, fig. 9). The plug has the same appearance in young and old sporangia (Pl. 3, fig. 5).

The structure of the young sporangium stimulated to germinate indirectly

Sudden exposure to a temperature of 13° for 1·5 hr followed by 10–15 min. at 22° (the stimulus for indirect germination) is sufficient to bring about several important structural changes. The central glycogen vacuole disappears and the cytoplasm contains a decreased number of smaller vacuoles which are now irregular in outline and possess structureless electron-transparent contents. Few peripheral vacuoles are recognizable. Mitochondria are enlarged and it is particularly noticeable that the cristae are elongated and penetrate deeply into the mitochondrial matrix (compare Pl. 5, figs. 11 and 12, with Pl. 2, fig. 3).

The sporangial wall shows a marked increase in complexity during the process of indirect germination. The outer structureless layer is unaltered, but the inner vesicular layer widens and, as germination advances, vesicular elements increase considerably in number and pack together to form a discrete, heterogeneous layer (Pl. 5, fig. 11). At this stage, moreover, a third layer is formed, separating the vesicular layer from the plasma membrane. This third and inner layer resembles the outermost layer of the wall in its low electron opacity and apparent absence of structure. It is conspicuous only in the walls of chilled sporangia, particularly when the changes are well advanced; there may, however, be indications of the beginning of a third layer in the apical wall of young non-germinating sporangia (Pl. 2, fig. 3).

The development of the papilla is even more striking. In the early stages of indirect germination the papilla is largely homogeneous and consists predominantly of a thickened outer structureless layer, although the vesicular layer and the innermost structureless layer are clearly detectable (Pl. 5, fig. 10). At a later stage the papilla presents a very different picture, with the vesicular layer now occupying the bulk of the central space (Pl. 5, fig. 12). The layer embodies a heterogeneous assortment of vesicles, some up to 1 μ in diameter and mostly containing moderately electron-opaque material; some less clearly defined vesicles of uniform size (0·5 μ) lie adjacent to the innermost cell wall and contain homogeneous material of lower electron opacity. The outer structureless layer is displaced outwards by the enlarged vesicular zone and a prominent bulge forms at the apex of the sporangium.

The elements comprising the plug remain unchanged during the process of indirect germination and the plug is not penetrated by the vesicular elements of the neighbouring wall.

DISCUSSION

During the process of ageing a sporangium of *Phytophthora erythroseptica* undergoes a series of irreversible changes which result in the loss of ability to germinate indirectly by the production of zoospores. Our electron microscope observations show that ageing is accompanied by a change in the appearance of the contents of the food vacuoles and by the disappearance of the vesicular layer in the wall. These changes suggest that the original food storage material (probably glycogen) and the vesicular layer of the wall both play essential roles in indirect germination. Two distinct processes are at work during indirect germination. The formation of zoospores involves the differentiation and cleavage of the sporangial cytoplasm.

Secondly, the sporangial wall must open in the region of the papilla tc permit the discharge of zoospores. The occasional release of sporangial contents before cytoplasmic differentiation is complete (Pethybridge, 1914; Waterhouse, 1962; Vujičić & Colhoun, 1966) indicates that the two mechanisms can operate independently even though they are normally stimulated by the same external conditions.

It is to be expected that the process of cytoplasmic differentiation will require an adequate source of energy and Yamamoto & Tanino (1961) showed that the glycogen content decreased and respiration increased during zoospore formation in sporangia of *Phytophthora infestans*. The results of the present study show that these physiological changes in *P. erythroseptica* can be correlated with corresponding changes in ultrastructure. Thus the disappearance of the large central vacuole together with the decrease in number and the change in appearance of the smaller vacuoles in chilled sporangia are consistent with a decrease in glycogen content. It is probable that the glycogen is converted into glucose and utilized as an energy source. The increase in respiration rate noted by Yamamoto & Tanino can be related to the changes in mitochondrial structure which occur after chilling. The short stumpy cristae in mitochondria of young non-germinating sporangia become elongated during indirect germination and penetrate deeply into the mitochondrial matrix; some increase in mitochondrial size is also apparent. Simon & Chapman (1961) noted that a rise in succinoxidase activity in developing Arum spadix cells was accompanied by a similar increase in the length of mitochondrial microvilli, while Hawker & Abbott (1963) noted that the number and length of mitochondrial cristae in vegetative hyphae of Rhizopus increased with hyphal age.

Discharge of the sporangial contents occurs on dissolution or rupture of the papilla. By this stage the sporangial wall of *Phytophthora erythroseptica* consists of three distinct layers: the outer wall (probably cellulose), the prominent vesicular layer and the innermost homogeneous layer. The striking development of the vesicular layer during indirect germination, particularly in the region of the papilla, and the emergence of the third layer separating the vesicular layer from the plasma membrane, are in marked contrast to the unaltered appearance of the outer sporangial wall. These observations provide clear evidence that the two inner layers are implicated in the discharge process and it may be postulated that the vesicles contain enzymes responsible for the ultimate degradation of papillar components.

Zoospores of Phytophthora may be liberated in various ways. The usual mechanism is by the discharge of zoospores as single mature motile organisms (Blackwell, 1949). In several species, however, for example in *Phytophthora cryptogea* (Pethybridge & Lafferty, 1919), *P. cactorum* (Wormald, 1919) and *P. colocasiae* (Blackwell & Waterhouse, 1930), zoospores may be expelled as a mass surrounded by a thin evanescent vesicle which remains attached to the mouth of the sporangium; in not more than a few seconds the zoospores break through the vesicle and swim away. In *P. cryptogea* the evanescent vesicle may be so tough that the zoospores must puncture a hole through which they escape singly (Blackwell, 1949). In the case of *P. erythroseptica* however the evanescent vesicle is apparently so delicate and transitory that it has only rarely been described (Pethybridge, 1914; Vujičić, 1963) and no trace of the vesicle remains after the dispersion of the zoospores. Light microscopy indicates that the evanescent vesicle is derived from the hyaline material

which comprises the papilla of the indirectly germinating sporangium (Pethybridge, 1914; Pethybridge & Lafferty, 1919; Wormald, 1919; Blackwell, 1949); the papilla has been described by Blackwell as a localized mucilaginous area of the inner sporangial wall which readily imbibed water and dissolved to allow emission of zoospores, sometimes giving rise to the evanescent vesicle during the process of solution. On the evidence provided by the present work it seems probable that this mucilaginous zone is the vesicular layer of the sporangial wall and that the evanescent vesicle is in some way derived from this layer.

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EXPLANATION OF PLATES

Abbreviations used

cv	central vacuole	*n*	nucleus
er	endoplasmic reticulum	*nm*	nuclear membrane
g	Golgi dictyosome	*np*	nuclear pore
hy	hypha	*p*	plug
lam	lamellar inclusion	*pa*	papilla
lip	lipid inclusion	*pm*	plasma membrane
m	mitochondrion	*pv*	peripheral vacuole
mc	mitochondrial cristae	*rib*	ribosomes

w_1 outer sporangial wall (homogeneous)
w_2 vesicular sporangial wall (heterogeneous)
w_3 inner sporangial wall (homogeneous)

PLATE 1

Fig. 1. Low magnification electron micrograph of a young sporangium of *Phytophthora erythroseptica* showing the central vacuole (*cv*) and the plug (*p*) connecting the sporangium to a hypha. Nuclei (*n*) are scattered in the cytoplasm. (Fixative OsO_4; ×2500.)

Fig. 2. Wall and cytoplasm of a young sporangium (from a 4-day-old culture). The sporangial wall consists of a thick homogeneous outer layer (w_1) and an inner vesicular layer (w_2) bordering the plasma membrane (*pm*). The nucleus (*n*) is enveloped by a double membrane (*nm*) penetrated by pores (*np*). Flattened cisternae and associated vesicles of a Golgi dictyosome (*g*), endoplasmic reticulum (*er*) and peripheral vacuoles (*pv*) are shown. (Fixative $KMnO_4$; ×80,000.)

PLATE 2

Fig. 3. The apical region of a young sporangium. The vesiculated layer (w_2) of the sporangial wall is well developed and the underlying plasma membrane (*pm*) shows numerous convolutions. Peripheral vacuoles (*pv*) are conspicuous in the outer regions of the cytoplasm; their finely granular contents are at places resolvable as small vesicles (arrows). Mitochondria (*m*) possess short stumpy cristae (*mc*) and extensive areas of clear matrix; elongated cisternae of the endoplasmic reticulum (*er*) occur in the vicinity of the nucleus (*n*). (Fixative $KMnO_4$; ×60,000.)

PLATE 3

Fig. 4. Lipid inclusions (*lip*) and inclusions with regions showing a periodic lamellar structure (*lam*) occur in the sporangial cytoplasm. Osmiophilic ribosomal particles (*rib*) are abundant. 8-day-old culture. (Fixative OsO_4; ×140,000.)

Fig. 5. The plug (*p*) separating the sporangium from the hypha (*hy*) in a young (4-day) specimen. (Fixative OsO_4; ×20,000.)

Fig. 6. Part of the central vacuole (*cv*) in a young (4-day) sporangium. (Fixative $KMnO_4$; ×90,000.)

PLATE 4

Fig. 7. Part of the central vacuole (*cv*) and adjacent cytoplasm in an old (10-day) sporangium. Mitochondria (*m*) and a lipid inclusion (*lip*) are shown. (Fixative $KMnO_4$; ×35,000.)

Fig. 8. Wall and cytoplasm of an old (10-day) sporangium. The wall consists only of the outer homogeneous layer (w_1); the inner vesiculated layer is absent. A nucleus (*n*) and nearby Golgi dictyosome (*g*) are shown. (Fixative $KMnO_4$; ×40,000.)

Fig. 9. Part of the papillar region in an old (8-day) sporangium. The papillae (*pa*) appear as a thick finely granular layer. (Fixative OsO_4; ×80,000.)

PLATE 5

Fig. 10. Part of the papillar region in a young chilled sporangium. In addition to the vesiculated layer (w_2), a third innermost layer (w_3) is detectable. (Fixative $KMnO_4$, ×30,000.)

Fig. 11. The wall of a chilled sporangium at an advanced stage of indirect germination. The three layers are clearly distinguishable with the vesicular layer (w_2) forming a wide and discrete region between the other two layers; the third and innermost layer (w_3) is particularly prominent. (Fixative $KMnO_4$; ×30,000.)

Fig. 12. The papilla of a chilled sporangium at an advanced stage of indirect germination. The greatly enlarged vesicular layer (w_2) now occupies the bulk of the central region of the papilla and displaces outwards the outermost homogeneous layer (w_1). Note the elongated cristae possessed by the mitochondria (*m*) in this and the two preceding figures. (Fixative $KMnO_4$; ×22,000.)

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Studies on the Haemagglutinin Present in Coxsackie A 7 Virus-Infected Suckling Mouse Tissue

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SUMMARY

Absorption and centrifugation studies established that the haemagglutinin specifically associated with suckling mouse tissue infected with Coxsackie A7 virus is particulate but distinct from the infectious particle. The haemagglutinin resembles other enteroviral haemagglutinins in being inactivated by *p*-chloromercuribenzoic acid. This property, together with other physical and chemical properties, suggests that the haemagglutinin is a protein. There is a serological relationship between the haemagglutinin and other non-haemagglutinating, serologically reactive substances present in tissues infected with Coxsackie A7 virus.

INTRODUCTION

Coxsackie A7 virus is outstanding among enteroviruses other than polioviruses as a cause of paralytic disease of man (Voroshilova, 1964; Rossi, Vassella & Rentsch, 1964; Grist & Bell, 1964). The virus resembles poliovirus in pathogenicity for man and monkey and shares with type 2 poliovirus a limited pathogenicity for adult rodents (Chumakov *et al.* 1956; Habel & Loomis, 1957; Grist & Roberts, 1962; Chlap & Lutynski, 1964). However, the Coxsackie virus is antigenically distinct from poliovirus and is pathogenic for day-old suckling mice, causing myositis characteristic of group A Coxsackie virus infection (Habel & Loomis, 1957; Voroshilova & Chumakov, 1959).

A strain of Coxsackie A7 virus was isolated during a study of an outbreak of poliomyelitis-like illness in Scotland in 1959 (Grist, 1960). Extracts of tissues from day-old suckling mice infected with the virus strain agglutinated vaccinia-agglutinable fowl red cells. Human, mouse and vaccinia-inagglutinable fowl red cells were not agglutinated. Haemagglutinins were not detected in similarly prepared extracts of uninfected suckling mouse tissue nor in those infected with other prototype group A Coxsackie strains (Grist, 1962). Haemagglutination was specifically inhibited by homologous antiserum. From these observations it appeared that the haemagglutinins detected were specifically associated with suckling mouse tissue infected with Coxsackie A7 virus.

The present paper reports an investigation of the relationship of the haemagglutinin to the infective particle and a comparison of the properties of the haemagglutinin with those of previously described viral haemagglutinins.

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METHODS

Virus. The virus used in this study was the 1034 strain isolated in 1959 from a paralysed child (Grist, 1960). This strain reacted as a type A7 strain related to both Dalldorf's prototype and the Russian AB-IV strain.

Preparation of infected tissue extracts. Day-old suckling mice were injected intraperitoneally with a dose of virus calculated to produce paralysis 4–5 days after injection. The carcasses were dissected to leave mainly skeletal muscle which was then homogenized with phosphate-buffered saline to give a 20% (w/v) suspension. Extracts were cleared by centrifugation and stored at $-20°$ until used. Before use, the extracts were thawed and centrifuged again to remove a flocculum, leaving a clear, straw-coloured supernatant fluid.

Haemagglutination tests. As a result of experiments to be described (see Results) the following procedure was adopted for routine haemagglutination tests. Blood from fowls with vaccinia-agglutinable red blood cells (r.b.c.) was collected aseptically by cardiac puncture into Alsever's solution. The r.b.c. were washed with physiological saline and used as a 0·5% suspension in saline. Cells not used immediately were stored in Alsever's solution at 4° until they showed signs of instability. Haemagglutination tests were carried out in plastic plates by addition of 0·3 ml. 0·5% r.b.c. to 0·3 ml. of twofold dilutions of 20% suckling mouse carcass suspensions in saline. End-points are expressed as reciprocals of the highest dilutions giving 3+ to 4+ haemagglutination by pattern formation.

Haemagglutination inhibition tests. One 100% haemagglutinin unit (final dilution of haemagglutinin giving 4+ haemagglutination pattern) was added in 0·3 ml. amounts to 0·3 ml. of twofold dilutions of immune serum and the mixtures held at room temperature for 1 hr. After this period, 0·3 ml. of 0·5% r.b.c. was added to each mixture and to the appropriate controls. Results were read when r.b.c. had completely settled and are expressed as reciprocals of the highest dilution of immune serum giving complete inhibition of haemagglutination.

Complement-fixation tests. The method employed was the standard small-volume, cold-overnight technique of this laboratory (Grist *et al.* 1965). In brief, tests were performed in plastic plates using 0·1 ml. amounts of each reagent consisting of serial twofold dilutions of antigen, 4 units of antiserum (determined by chess-board titration) and 4 units of complement. After overnight fixation at 4°, plates were incubated at 37° for 20 min. and 0·1 ml. of 2% sensitized sheep cells added. Lysis was allowed to proceed at 37° for 40 min. and the plates then kept at 4° until cells had settled. End-points are expressed as the reciprocal of the highest dilution of antigen giving complete or nearly complete fixation of complement.

Infectivity titrations. Serial tenfold dilutions of infective preparations were injected in 0·03 ml. amounts intraperitoneally into day-old suckling mice. A litter of at least seven animals was used for each dilution. The mice were observed for a total of 14 days for signs of paralysis; mice dying within 24 hr of injection were rejected from the titration. The effective dose at which 50% of the animals injected showed paralysis (ED 50) was determined by the Kärber formula. Infectivity titres are expressed as the log. of the reciprocal of the ED 50 end-point ($\log_{10}$ ED 50/0·03 ml.).

Neutralization tests. Serial twofold dilutions of immune serum were mixed with an

equal volume of virus preparation containing 100 ED50/0·03 ml. and held at room temperature for 2 hr. The virus serum mixtures were then injected in 0·03 ml. amounts intraperitoneally into day-old suckling mice using a litter of at least seven animals for each dilution. Serum neutralization titres are expressed as reciprocals of 50% end-points determined by the Kärber formula.

Preparation of antiserum. Adult mice were immunized by four intraperitoneal injections at weekly intervals of 0·5 ml. amounts of 20% suspensions of carcasses from suckling mice infected with the 1034 strain. Seven days after the fourth injection, immunized mice were injected intraperitoneally with 0·2 ml. Landschütz ascites tumour fluid and the resultant immune ascitic fluid harvested 7 days later. Immune ascitic fluid was used for routine complement-fixation tests. Serum absorption experiments were carried out using immune serum obtained from immunized adult mice by cardiac puncture.

RESULTS

Optimal conditions for demonstration of haemagglutination

Salt. Extracts of infected tissue were dialysed against distilled water overnight and haemagglutination titres determined using various isotonic salt solutions as diluent and for preparation of 0·5% r.b.c. suspensions. Haemagglutination was not detectable when haemagglutination tests were carried out in isotonic solutions of glucose. Identical haemagglutination titres were obtained in isotonic solutions of NaCl, $MgCl_2$, $CaCl_2$ and Na_2SO_4.

Haemagglutination by extracts of Coxsackie A7 virus-infected tissues is dependent, therefore, on the presence of salts. Since identical haemagglutination titres were obtained in media containing either only monovalent anions or cations or only divalent anions or cations, the valency of the salts in the medium was not critical.

Temperature. Haemagglutination titres up to 256 were obtained when tests were made at room temperature (15–18°) and parallel tests showed a twofold decrease at 4° and a twofold increase at 37° compared with titres obtained at room temperatures. The pattern of agglutinated cells remained stable for 24 hr at each temperature.

Freshly prepared extracts of infected tissues, however, caused considerable haemolysis after prolonged incubation. Haemolysis was also demonstrable with vaccinia-inagglutinable r.b.c. and lysins were found in similarly prepared extracts of uninfected suckling mouse tissue. Haemolysis of vaccinia-agglutinable r.b.c. by extracts of infected tissue was not inhibited by 1034-immune serum. Thus haemolysis is not specifically associated with Coxsackie A7 virus-infected tissue but is due to lysins known to be present in homogenates of normal mouse tissue (Herberman, 1964).

pH. Haemagglutination titres were determined using diluents of different pH compatible with the stability of both the haemagglutinin and fowl r.b.c. Experiments were made at room temperature using physiological saline buffered with citrate buffer, pH 4·5–6·0, phosphate buffer, pH 7·0–8·0, and glycine buffer, pH 9·0–10·5.

Maximal haemagglutination titres were obtained between pH 4·5 and pH 8·0 but without a well-defined pH optimum. Increased alkalinity produced a progressive decrease in haemagglutination titre with a 32-fold decrease in titre at pH 11·0 compared with the maximal titres. As a result of these experiments, haemagglutination

tests were routinely carried out at room temperature as described in Methods. The pH of physiological saline used as a diluent was between 6·4 and 6·8.

Relationship of the haemagglutinin to the infective particle

Absorption studies. Extracts of tissue infected with Coxsackie A7 virus were absorbed at room temperature for 2 hr with 5% suspensions of vaccinia-agglutinable and vaccinia-inagglutinable fowl r.b.c. The mixtures were kept at 4° overnight and the supernatant fluids removed after deposition of the r.b.c. by centrifugation. The deposited cells were washed with saline at 4°, resuspended in saline to volumes equivalent to the original absorption mixtures and disrupted by two cycles of freezing and thawing. Supernatant fluids and suspensions of disrupted cells were assayed for infectivity, haemagglutinating and complement-fixing activity.

Table 1. *Absorption of Coxsackie A7 virus-infected suckling mouse tissue extract with vaccinia-agglutinable and vaccinia-inagglutinable fowl r.b.c.*

Expt. no.	Preparation	Titres* HA	CF	INF
1	Unabsorbed extract	128	64	6·9
2	Absorbed with vaccinia-agglutinable fowl r.b.c.	0	64	6·8
3	Absorbed with vaccinia-inagglutinable fowl r.b.c.	128	64	6·8
4	Lysate of vaccinia-agglutinable fowl r.b.c. from Expt. 2	—	0	2·0
5	Lysate of vaccinia-inagglutinable fowl r.b.c. from Expt. 3	—	0	1·9

* HA, haemagglutination; CF, complement fixation; INF, infectivity = $\log_{10}$ ED 50/0·03 ml.

Haemagglutinins were removed specifically from the infective extracts by absorption with vaccinia-agglutinable fowl r.b.c. (Table 1). Loss of haemagglutinating activity was not accompanied by any detectable decrease in infectivity titre. Owing to the lack of precision of the method used for infectivity titration this result does not exclude the possibility that haemagglutinating activity is associated with a small proportion of the total population of infective particles. However, such an association should have been revealed by a higher infectivity titre in the lysate of vaccinia-agglutinable fowl r.b.c. compared with that of vaccinia-inagglutinable fowl r.b.c. No such difference was detected. These results strongly suggest, therefore, that the haemagglutinin and infective particle are distinct entities.

In addition, there was no detectable decrease in complement-fixing activity of the infected tissue extracts compared with that of controls after complete removal of the haemagglutinins. Within the limits of sensitivity of the serological test, therefore, the haemagglutinin does not appear to be associated with complement-fixing activity.

Centrifugation studies. In order to investigate further the relationship of the haemagglutinin to the infective particle, extracts of tissues infected with Coxsackie A7 virus were examined by centrifugation. A saline extract of infected tissue was centrifuged repeatedly for constant periods of one hour at 4° at increasing centrifugal force in the no. 40 rotor of a Spinco Model L ultracentrifuge. After each period of centrifugation, samples were removed from the centre of the centrifuge tube. The infectivity, haemagglutinating and complement-fixing titres of each successive sample were determined (Fig. 1).

The rate of sedimentation of haemagglutinating activity under the conditions described is thus greater than that of infectivity, confirming the conclusion drawn from the absorption experiments that the haemagglutinin and infective particle are distinct entities. Further, these studies show that the haemagglutinin is particulate and not a 'soluble substance'. Both the haemagglutinin and infective particle were found at the same level in equilibrium density gradient centrifugation experiments in caesium chloride (Grist, unpublished results). These experiments suggest that the haemagglutinin is a larger particle than the infective particle.

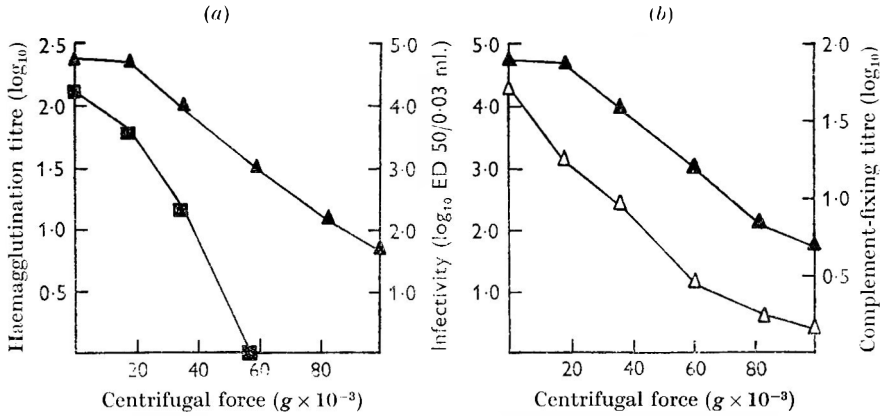

Fig. 1. Relative rates of sedimentation of infectivity, haemagglutinating and complement-fixing activities associated with extracts of Coxsackie A7 virus-infected suckling mouse tissue. (*a*) ▲—▲ = infectivity; ■—■ = haemagglutinating activity. (*b*) ▲—▲ = infectivity, △—△ = complement-fixing activity.

Unlike haemagglutinating activity, the rate of sedimentation of complement-fixing activity closely paralleled that of infectivity apart from the initial stages. Complement-fixing activity is mainly associated, therefore, with the infective particle. It is not possible to draw firm conclusions from the initial rate of sedimentation of complement-fixing activity which might suggest that this activity is independent of the infective particle.

Effect of physical and chemical agents on the Coxsackie A 7 haemagglutinin

Stability of the Coxsackie A7 haemagglutinin was tested under experimental conditions described in a systematic study of virus haemagglutinins by Buckland & Tyrrell (1963). The results provide information relevant to the probable chemical nature of the haemagglutinin and its relationship to previously described virus haemagglutinins.

Coxsackie A7 haemagglutinin was completely stable over a range from pH 2 to 10. Although Coxsackie viruses were not examined by Buckland & Tyrrell, it is perhaps significant that those enteroviruses studied were found to be stable over a similar pH range. Haemagglutinating activity was lost after heating at 56° for 60 min. and after exposure to 4M-urea or 0·005M-*p*-chloromercuribenzoic acid. Inactivation by *p*-chloromercuribenzoic acid was reversible by subsequent treatment of the inactivated haemagglutinin with 0·5M-cysteine or glutathione at 37° for 30 min. These results, together with the precipitation of haemagglutinating activity

by 25% ammonium sulphate, are compatible with a proteinaceous haemagglutinin. However, haemagglutinating activity was not affected by treatment with 1% papain, 0·01% α-chymotrypsin or 0·01% trypsin. Treatment with papain for longer periods or with 0·1% solutions of the two other enzymes at 37° for 4 hr resulted in a consistent twofold increase in haemagglutination titre. An increase in haemagglutination titre was also described following treatment of vaccinia-infected tissues with trypsin (Briody, 1951; Youngner & Rubinstein, 1962).

Evidence against the association of haemagglutinating activity with either carbohydrate or lipid is provided by resistance to 0·046M-potassium periodate and organic solvents in which the haemagglutinin is also insoluble. Nor was there detectable decrease in haemagglutinating titre after incubation at 37° for up to 48 hr with cobra venom. When examined under similar conditions, vaccinia haemagglutinin, prepared from vaccinia-infected chick embryo chorioallantoic membrane, was almost completely inactivated. Inactivation of vaccinia haemagglutinin by cobra venom is considered to be due to a phospholipase (Stone, 1946).

Serological studies

Absorption and centrifugation studies established the discrete nature of the Coxsackie A7 haemagglutinin. It seemed of interest, therefore, to determine whether there was any serological relationship between the haemagglutinin and the other serologically reactive materials present in extracts of Coxsackie A7 virus-infected tissue.

A haemagglutinin-free preparation was obtained by exhaustive absorption of an infected tissue extract with vaccinia-agglutinable fowl r.b.c. This preparation was then used to absorb Coxsackie A7-immune mouse serum by mixing equal volumes of haemagglutinin-free preparation and immune serum and incubating the mixtures at 37° for 2 hr and then keeping at 4° overnight. In order to increase the sensitivity of the test, the immune serum was diluted to 16 times the complement-fixing, haemagglutination inhibiting and neutralization titres before addition of the haemagglutinin-free preparation. After absorption, virus was removed by centrifugation at 40,000 rev./min. for 4 hr in the no. 40 rotor of a Spinco Model L ultracentrifuge.

Table 2. *Absorption of Coxsackie A7-immune mouse serum with a haemagglutinin-free extract of Coxsackie A7 virus-infected suckling mouse tissue*

	Serum titres*		
Antisera	HI	CF	VN
Unabsorbed serum	512	128	2048
Absorbed serum	128	< 32	< 512
Normal serum	< 64	< 32	< 512

* HI, haemagglutination; CF, complement fixation; VN, virus neutralization.

Absorption of immune serum with the haemagglutinin-free preparation resulted in a marked decrease in complement-fixing and neutralization titres. This result was anticipated in view of the association of complement-fixing activity with the infective particle which was established by centrifugation studies. However, these reductions in titre were also accompanied by a significant specific decrease in

haemagglutination-inhibition titre (Table 2). This result establishes that there is a serological relationship between the haemagglutinin and the other serologically reactive materials present in extracts of Coxsackie A7 virus-infected tissue.

DISCUSSION

Haemagglutinins specifically associated with enterovirus-infected tissues previously described may be classified in two groups. First, there are those in which haemagglutination is a property of the infective particle (Goldfield, Srihongse & Fox, 1957). Under appropriate conditions, passage of such viruses may give rise to a mixed population of haemagglutinating and non-haemagglutinating virions (Johnson & Lang, 1962). Absorption of extracts of tissues infected with such viruses with susceptible r.b.c. results in loss of haemagglutinating activity with concomitant complete or partial loss of infectivity.

The second group consists of those enteroviruses which infect tissues producing two types of haemagglutinin, one associated with the infective particle and the other non-infective haemagglutinin (Fabiyi, Engler & Martin, 1964). Physical examination has shown that this second haemagglutinin is a particle of lower density than the infective particle and lacks the typical icosohedral symmetry of the virion. It is probable that the non-infective haemagglutinin is an 'incomplete' or 'coreless' form of the infective particle.

Absorption of Coxsackie A7 virus-infected tissue with vaccinia-agglutinable fowl r.b.c. removed the haemagglutinin without any detectable decrease in infectivity. Within the limits of sensitivity of the infectivity titration technique employed, these results show that the haemagglutinating activity is not associated with the infective particle. The results of density gradient centrifugation experiments suggest that the haemagglutinin and infective particle have the same density. It is unlikely, therefore, that the Coxsackie A7 haemagglutinin is an 'incomplete' virus particle. Indeed, differential centrifugation experiments suggest that the haemagglutinin is larger than the infective particle.

The Coxsackie A7 haemagglutinin also differs from previously described enteroviral haemagglutinins in not agglutinating human group O cells. However, its inactivation by *p*-chloromercuribenzoic acid and subsequent reactivation by thiol compounds is a property typical of enteroviral haemagglutination (Buckland, 1960; Philipson & Choppin, 1960).

Another paradoxical property of the Coxsackie A7 haemagglutinin is its reactivity with vaccinia-agglutinable fowl r.b.c. Vaccinia haemagglutinin agglutinates only those fowl cells which are susceptible to agglutination by lipid suspensions (Burnet & Stone, 1946); this property, together with the inactivation of vaccinia haemagglutinin by cobra venom and other preparations known to contain phospholipases, is a strong indication that the activity of the vaccinia haemagglutinin is associated with phospholipid (Stone, 1946). The physical and chemical studies described, however, suggest that the activity of the Coxsackie A7 haemagglutinin is not associated with lipid but with protein. The differing chemical nature of the haemagglutinins suggests that the vaccinia and Coxsackie A7 haemagglutinins attach to susceptible fowl r.b.c. by different receptors. This hypothesis is at present under investigation.

The significance of the production of haemagglutinins in Coxsackie A7 virus-infected suckling mouse tissue is still a matter for conjecture. Two main alternatives present themselves for the possible origin of the haemagglutinin: the haemagglutinin either is derived from normal host-cell constituents or else is synthesized *de novo* through the metabolic pathways of the virus-infected cells. Failure to detect similar haemagglutinins in similarly prepared extracts of uninfected tissues or in tissues infected with other Coxsackie viruses suggests that the active principle of haemagglutination is not a component of uninfected tissues. Since specific haemagglutinins have also been detected in Coxsackie A7 virus-infected tissue of a different rodent species, the adult cotton rat (Grist, 1962), production of haemagglutinins is not a phenomenon peculiar to suckling mouse tissue. Serological studies have established that the haemagglutinin is composed, in part at least, of virus-specific material. Further, it has been shown that antibodies specifically inhibiting haemagglutination cross-react with a haemagglutinin-free fraction of infected tissue extract. A common serological specificity must be shared, therefore, by both the haemagglutinin and other non-haemagglutinating virus-specific material. This suggests that the haemagglutinin carries two reactive sites only one of which is responsible for the haemagglutinating activity.

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