Ultrastructure of Sclerotia and Hyphae of *Sclerotium rolfsii* Sacc.

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SUMMARY

The ultrastructure of cells of the sclerotia and aerial mycelium of *Sclerotium rolfsii* Sacc. was studied by electron microscopy. A mature sclerotium of *S. rolfsii* contains several types of differentiated cells. The thick-walled rind cells which comprise the sclerotial envelope are empty. The underlying cortex cells have thinner walls and contain many vesicles full of reserve materials which appear dark after treatment with osmium tetroxide. Although less reactive to osmium tetroxide, the cells between the cortex and medulla are also rich in reserve materials, some of which are membrane-bound. The inner layer (medulla) is composed of cells with extremely thick walls, of thinner-walled cells full of reserve materials and of empty cells. The walls of the hyphal cells are significantly thinner, and less optically dense than the walls of any of the sclerotial cells. Hyphal cells contain more ribosomes and mitochondria than sclerotial cells.

It seems that the resistance of sclerotia to biological degradation depends upon the melanin-rich rind as well as the wall structure and organization of cells comprising the inner layers of the sclerotium.

INTRODUCTION

Some septate fungi are capable of forming sclerotia, which consist of firm aggregates of vegetative hyphae and serve as a means of survival for these fungi, being very resistant to biological degradation (Butler, 1966).

According to Townsend & Willetts (1954), who investigated the development and the structure of several types of sclerotia by the light microscope, a mature sclerotium of *Sclerotium rolfsii* Sacc. is composed of four distinct cell layers: (*a*) a fairly thick skin, (*b*) a rind, two to four cells thick, made of broad and tangentially flattened cells, (*c*) a cortex of thin-walled cells with densely staining cytoplasm and (*d*) a medulla made of loosely arranged, ordinary filamentous hyphae, also filled with dense contents. So far, no information is available about the morphology and function of individual cells comprising these layers. The present study was begun in an attempt to reveal the organization and ultrastructure of the sclerotial cells, as compared with hyphal cells of *S. rolfsii*.

METHODS

The fungus *Sclerotium rolfsii* Sacc. was isolated from infected sugar beet. It was grown in Petri dishes, each containing 20 ml. of defined medium (Chet, Henis & Mitchell, 1967) prepared according to Joham (1943). The inoculated agar plates were incubated at 30°.

Samples of aerial mycelium were taken from the centre of a 3-week-old colony ('old' mycelium) and from the margins of a 5-day-old colony ('young' mycelium).

Thin sections for electron microscopy were prepared from brown, mature sclerotia or aerial hyphae. These were dipped in tubes, each containing 1 ml. cooled 5% glutaraldehyde in 0·1 M-phosphate buffer (pH 7·0) for 1 hr. After three rinsings with cold buffer the material was fixed with cold 2% OsO_4 in the same buffer for 22 hr (sclerotia) or for 2 hr (hyphae). After dehydration with ethanol the material was embedded in Epon 812. Thin sections were prepared with an LKB Ultrotome III, using glass knives. The sections were stained with 3% uranyl acetate for 1 hr with 'Karnovsky's Method A' lead hydroxide (Pease, 1964), for 20 min. and examined with a JEM-T7 electron microscope, and with a Zeiss GFL phase-contrast microscope.

RESULTS

Sclerotia

A phase contrast micrograph of a section of a mature sclerotium is seen in Pl. 1, fig. 1. The layers described by Townsend & Willetts (1954) are clearly seen. The rind (a) was composed of thick-walled, empty cells. The cortex cells (b) appeared very dark, possibly as a result of a reaction of their polymetaphosphates or lipids with OsO_4 (Bracker, 1967; Edwards & Edwards, 1960; Scurti & Converso, 1965). In addition, an intermediate layer (b_1), containing cells with brightly granulated cytoplasm was seen between the cortex and the medulla (c).

In electron micrographs of sections of sclerotia, the walls of all sclerotial cells (Pl. 1, fig. 2 to 6; Pl. 2, fig. 7 and 8) appeared thicker than those of the hyphae (Pl. 2, fig. 9 to 14). Cell wall residues, apparently comprising a part of the sclerotial skin (Townsend & Willetts, 1954), were attached to the melanin-rich, thick-walled rind cells (Bloomfield & Alexander, 1967) which appeared empty (Pl. 1, fig. 2). The cortex cells (Pl. 1, fig. 3) were very big and were full of huge vesicles, leaving only a little space for the cytoplasm and other cell organelles.

The cells of the intermediate layer (Pl. 1, fig. 1, b_1 were of special interest. Their walls were thicker than those of the cortex cells, their cytoplasm was full of electron-dense granules (Pl. 1, fig. 4 and 5, g) typical of polysaccharides (Bracker, 1967) and of membrane-bound electron-dense bodies (d) similar to those described by Gay & Greenwood (1966).

In contrast to the above layers, the cells comprising the medulla (Pl. 1, fig. 1 c; fig. 5) were loosely arranged and extremely heterogeneous, showing empty thin-walled cells (Pl. 1, fig. 5, arrow) and extremely thick-walled cells (Pl. 1, fig. 5 and 6; Pl. 2, fig. 7) resembling 'fibre hyphae' found in rhizomorphs of *Armillaria mellea* and *Trametes quercina* by Schmidt & Liese (1968), as well as cell types similar to those found in the intermediate layer of the cortex. Dolipore septa characteristic of basidiomycetes (Bracker, 1967) were also observed (Pl. 1, fig. 5; Pl. 2, fig. 8).

Hyphae

In both 'young' (Pl. 2, fig. 9 to 11) and 'old' (Pl. 2, fig. 12 to 14) cell types the wall was much thinner than that of any sclerotial cell. In contrast to the sclerotial cell walls, the fibrillar structure of the hyphal walls was clearly seen (Pl. 2, fig. 12 and 14). The hyphal wall thickness varied from 0·1 μ at the tips to 0·3 μ. In both 'old' and

'young' hyphae, cytoplasmic membrane was significantly folded in the hyphal tips (Pl. 2, fig. 9 and 10). In addition, small vesicles or tubules closely associated with the cytoplasmic membrane were observed in most sections. These structures were not observed in the sclerotial cells. They seemed to be enclosed between the cytoplasmic membrane and the cell wall (Pl. 2, fig. 11 to 14).

Dark bodies, possibly polysaccharides (Bracker, 1967), appeared especially in the vicinity of hyphal tip initiation (Pl. 2, fig. 9 and 10). In general, hyphal cells were much richer in ribosomes and mitochondria than sclerotial cells, indicating a lower metabolic activity of the sclerotial cells.

DISCUSSION

The results presented here confirm that the sclerotium of *Sclerotium rolfsii* is a complex body, containing differentiated and possibly specialized cell types (Aycock, 1966). The description of the ultrastructure of the cortex and the medulla of sclerotia of *S. rolfsii* differs from that reported by Townsend & Willetts (1954), who assumed that both layers consisted of ordinary hyphal mycelium.

The structure of the hyphal cells of *Sclerotium rolfsii* as observed under the electron microscope is very similar to that described by Icochea (1966), who used phase microscopy and staining procedures. In addition to the usual subcellular organelles and reserve materials, hyphal cells often contain small vesicles attached to the cytoplasmic membrane, resembling 'lomasomes', considered by some authors as playing a role in cell wall synthesis (Bracker, 1967), or 'multivesicular bodies' found by Calonge, Fielding & Byrde (1969) in *Scelorinia fructigena*, possibly playing a role in enzyme secretion.

It is of interest to compare the structure of sclerotia of *Sclerotium rolfsii* to those of other fungi. The structure of microsclerotia of *Verticillium albo-atrum* was investigated by Nadakavukaren (1963), who found that microsclerotial units were composed of thin- and thick-walled cells in close association with each other. The large thick-walled cells contained different cytoplasmic inclusions and food vacuoles, whereas the thin-walled cells contained nuclei or were empty. The thick-walled cells appeared to serve both as a protection and as a nutrient source for the thin-walled ones.

Scurti & Converso (1965), who studied the ultrastructure of sclerotia produced by a *Typhula* sp., noted the presence of two cell types: (*a*) cells with high metabolic activity; (*b*) cells rich in stored food material. Cytoplasm of the first type was abundant and homogeneous, containing mitochondria and endoplasmic reticulum. Cells of the second type had thicker walls and their nuclei and mitochondria were hidden beneath the stored food material, which consisted of polymetaphosphates, polysaccharides and fats.

The findings presented in this study support the earlier suggestions that melanin alone does not account for the higher resistance to lytic enzymes of sclerotial as compared to hyphal walls (Chet & Henis, 1967, 1969).

It seems that the studies hitherto made on the chemical composition of sclerotial walls either represent the sclerotial skin rather than the outer rind as claimed by Bloomfield & Alexander (1967), or an average value of the walls of aggregates of different cell types, mainly from the cortex and the rind (Chet *et al.* 1967), whose separation seems impossible at present. Therefore, the interesting problem of the

specific role of the various cell types in sclerotial persistence and germination awaits further investigation.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. A thin section of a mature sclerotium fixed with osmium tetroxide, showing rind (a), dark cortex (b), intermediate layer consisting of cells with brightly granulated cytoplasm (b_1) and medulla (c). Phase contrast ($\times$ 1000).

Fig. 2. Ultrathin section of the outer layer of a mature sclerotium showing skin (arrows) and empty rind cells with thick walls (w) ($\times$ 7500).

Fig. 3. Ultrathin section showing parts of some cortex cells, with thick walls (w) and huge vesicles (v) full of fine, dark granules ($\times$ 7500).
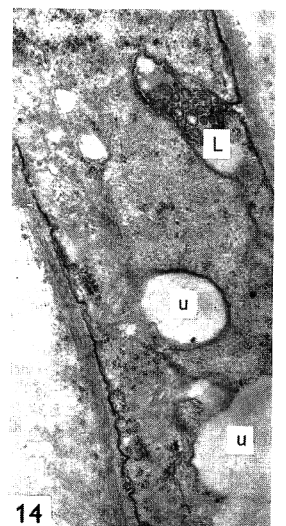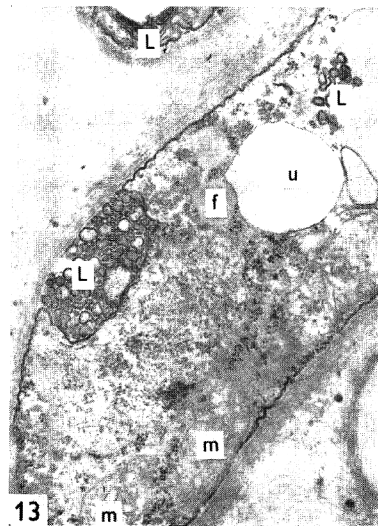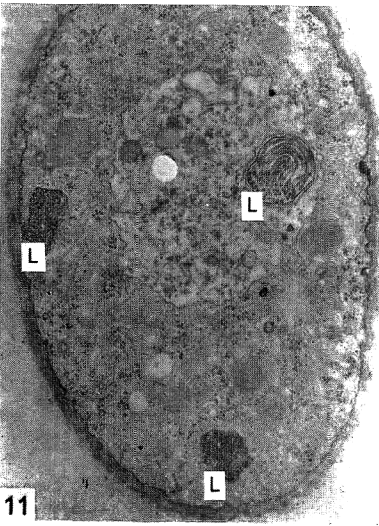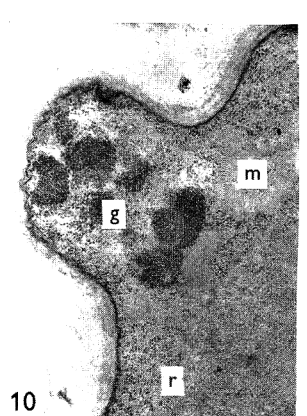
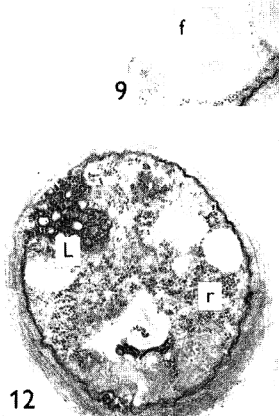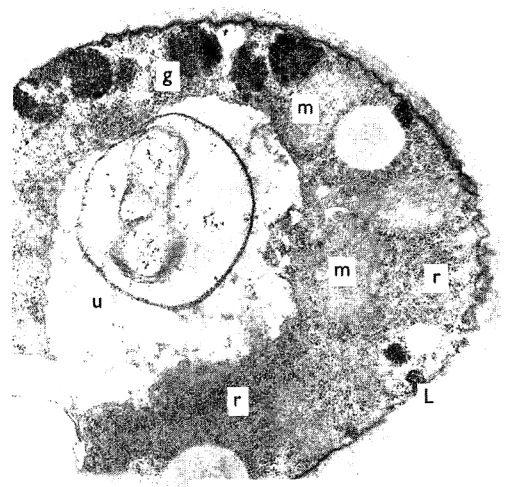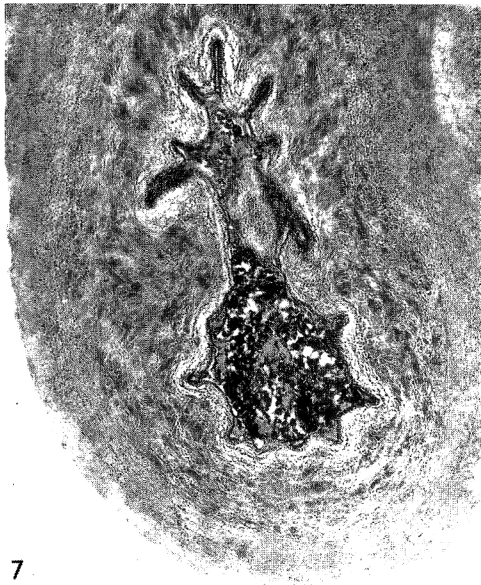I. CHET. Y. HENIS AND N. KISLEV

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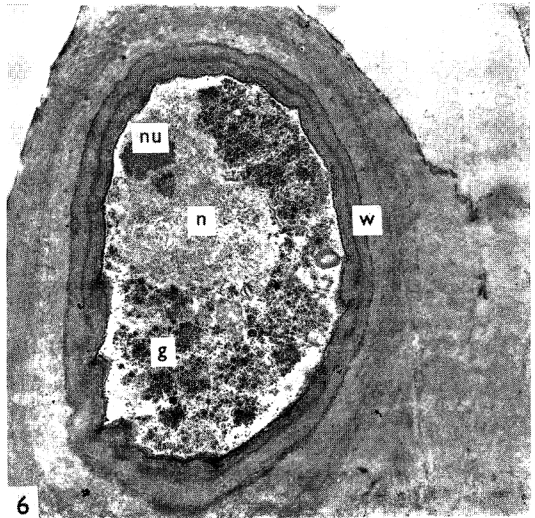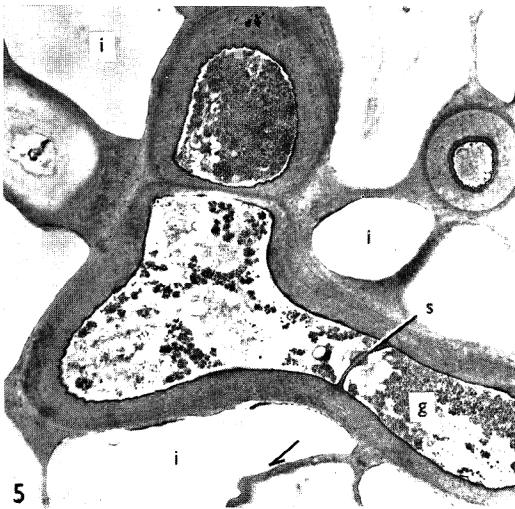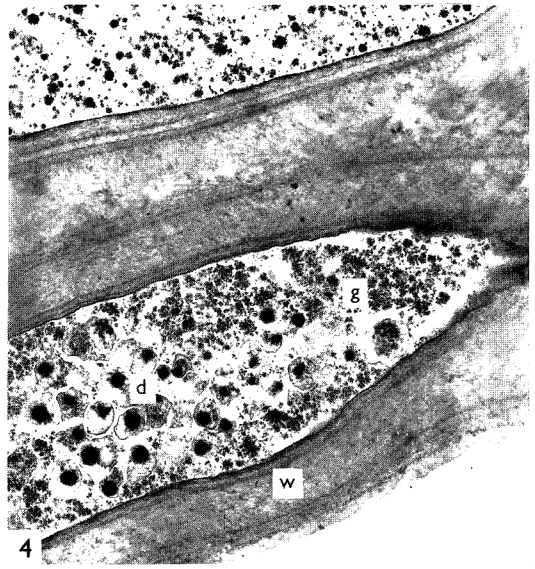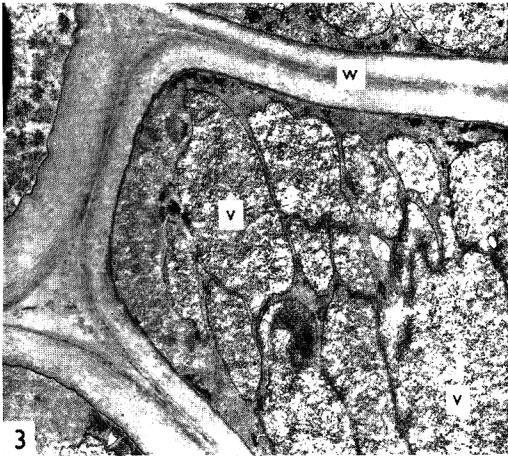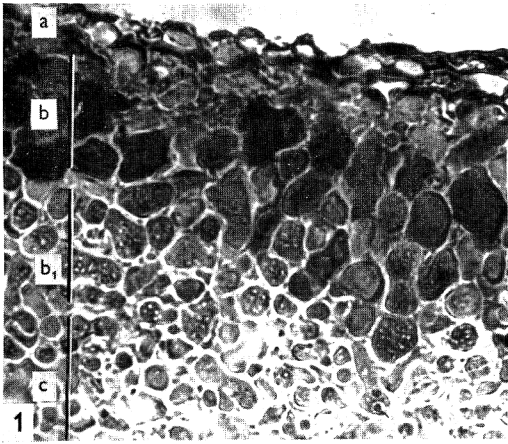
Fig. 4. A part of a cell from the intermediate layer (b₁) (Fig. 2) showing thick wall (w), polysaccharide granules (g) and membrane-bound electron dense bodies (d) (× 7500).

Fig. 5. Ultrathin section of medullar cells, showing cells with thick wall (w), full of polysaccharide granules (g), empty cells with thin walls (arrows), a dolipore septum (s) connecting separate cells and intracellular spaces (i) (× 3400).

Fig. 6. Ultrathin section of a thick-walled medullar cell, showing a thick wall (w) consisting of several layers, polysaccharide granules (g), nucleus (n) and nucleolus (nu) (× 7500).

PLATE 2

Fig. 7. A medullar cell with extremely thick wall and degenerated cytoplasm (× 12500).

Fig. 8. Ultrathin section of two adjacent medullar cells showing thick walls (w), dolipore septum (s), polysaccharide granules (g) and membrane-bound, electron-dense bodies (d) (× 7500).

Fig. 9. Ultrathin section of a hyphal tip of a 'young' aerial mycelium, showing thin wall and folded cytoplasmic membrane, polysaccharide granules (g), mitochondria (m), 'lomasomes' (L), fat bodies (f) and a vacuole (u). Note the abundance of ribosomes (r) (× 15000).

Fig. 10. A branching initiation at the distal part of the same hyphal tip shown in Fig. 9. Note the concentration of polysaccharide granules (g) at the growing tip. Symbols as in Fig. 9. (× 15000).

Fig. 11. Ultrathin transverse section of a 'young' hyphal cell, showing groups of tubular 'lomasomes' (L), some being cut longitudinally and some transversely. One group is observed between the cell wall and the cytoplasmic membranes; others appearing as situated inside the cell, probably protruding from the cell boundary (see also Fig. 14) (× 15000).

Fig. 12. Ultrathin section of 'old' hyphal cells, showing 'lomasomes' (L) and ribosomes (r) (× 15000).

Fig. 13 and 14. Longitudinal section of tip (Fig. 13) and distal (Fig. 14) parts of 'old' hyphal cells, showing mitochondria (m), fat bodies (f), 'lomasomes' (L) and vacuole (u) (× 15000).

Mechanisms of Inhibition of Fungi in Agar by Streptomycetes

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SUMMARY

Cf 20 unidentified Streptomyces isolates tested, all inhibited *Mucor ramannianus* and 18 inhibited *Glomerella cingulata* in agar. Agar discs from inhibition zones, or paper discs placed beneath inhibition zones, of nine of the Streptomyces isolates caused new inhibition zones when transferred to fresh, seeded agar plates. Transferable inhibition zones were not produced by the other 11 isolates. Inhibitory substances were produced in liquid cultures by eight of the nine isolates which produced them in agar media, whereas no antibiotics were detected in liquid cultures of the other 11 isolates. *Glomerella cingulata* conidia, which do not require exogenous nutrients for germination, germinated in liquid cultures of non-antibiotic-producing streptomycetes, but failed to germinate in cultures of antibiotic-producing streptomycetes. Inhibition zones produced by non-antibiotic streptomycetes decreased in size with increasing concentration of nutrients, whereas those of antibiotic streptomycetes were unchanged. Glucose and glutamic acid levels in agar rapidly decreased adjacent to streptomycete colonies. Agar, leached of nutrients by sterilized distilled water running slowly through a groove cut in the agar, developed clear inhibition zones. Therefore, inhibition of fungi by streptomycetes in agar, in some cases, appears to be due to nutrient deprivation.

INTRODUCTION

Many streptomycetes inhibit fungi and other micro-organisms in artificial media. However, about half of those which show antagonism on solid media fail to produce any detectable inhibitory substances in liquid media containing the same nutrient materials (Florey *et al.* 1949; Schatz & Hazen, 1948; Stessel, Leben & Keitt, 1953; Waksman, 1945; Waksman, 1967). The reasons for this are not understood, but it is generally assumed that cultural conditions are somehow less favourable for antibiotic production in liquid than on solid media (Florey *et al.* 1949).

Recent information from this laboratory indicated that fungal spores may fail to germinate in soil because micro-organisms deplete nutrients rather than form inhibitory substances (Lockwood, 1964; Ko & Lockwood, 1967). Among other lines of evidence, the inhibition of spore germination on agar placed in contact with soil correlated with the loss of nutrients from the agar rather than with the diffusion of fungistatic materials into the agar. The purpose of this study was to investigate the mechanisms of the inhibition of fungi by streptomycetes in agar and particularly to determine if nutrient depletion might be a mechanism of antago.

METHODS

Preparation of streptomycetes and fungi. Twenty unidentified Streptomyces isolates from soil and the test fungi, *Glomerella cingulata* and *Mucor ramannianus*, were maintained on potato glucose agar. Conidia of *G. cingulata* required no exogenous nutrients for germination, whereas those of *M. ramannianus* required an exogenous carbon source. Conidia were removed from the surface of agar slopes with sterilized distilled water.

Assays of inhibition zones. Tests for inhibitory activity were made in Petri dishes on peptone agar (per litre: peptone, 5 g.; agar, 20 g.) and peptone glucose agar (per litre: peptone, 5 g.; glucose, 5 g.; agar, 20 g.). Streptomycetes were streaked on the media, and incubated for 2 or 3 days at 24°. Then conidial suspensions of *Glomerella cingulata* or *Mucor ramannianus* were sprayed on to the agar surface with a modified Stansly's spray apparatus (Johnson, Curl, Bond & Fribourg, 1959). Approximately $2 \cdot 5 \times 10^5$ spores were sprayed on to each plate. After 3 days the inhibition zones were measured.

The presence of antibiotics in the inhibition zones was tested by three methods. (i) Agar discs, 7 mm. in diam., were cut from inhibition zones and transferred to the surface of peptone agar and peptone glucose agar, or to Petri dishes without agar, and allowed to incubate without supplying additional inoculum of the test fungus. Presence or absence of growth of the test fungus was observed. (ii) Agar discs, 7 mm. in diam., were cut from inhibition zones and transferred to the surface of peptone agar and peptone glucose agar. Immediately or several hours later a spore suspension of the same test fungus was sprayed on to the agar surface. The presence or absence of new inhibition zones was observed. (iii) Sterile filter paper discs, 13 mm. in diam., were placed beneath the agar of the inhibition zones for 10 to 15 days to allow any inhibitory substances to diffuse into the paper discs. The discs were then transferred to the surface of peptone agar and peptone glucose agar, and the test fungus was sprayed on the agar surface. The development of any new inhibition zones was observed.

Assays of lytic zones. Mycelia of *Glomerella cingulata* were prepared by mixing conidia with warm (42°) peptone agar at the rate of 4000/ml. The agar was then poured into Petri dishes and incubated for 2 to 3 days. Mycelia grew primarily within the agar, producing a dense, uniform growth with relatively few appressed hyphae on the surface. Streptomyces isolates were then streaked on the surface of the agar. At 10, 25 and 30 days, agar discs, 7 mm. in diam., were cut from lytic zones and transferred to peptone agar and peptone glucose agar. A spore suspension of *G. cingulata* was sprayed on to the surface of the agar to test for the presence of diffusible inhibitory substances. Other discs were transferred to peptone agar containing growing mycelium of *G. cingulata* to test for diffusible lytic substances.

Detection of antibiotics. Streptomycetes were cultured in 125 ml. Erlenmeyer flasks containing 20 ml. liquid medium per flask. The media were: (i) Bacto (Difco) nutrient broth, 6 g.; glucose, 5 g.; NaCl, 5 g.; $ZnSO_4.7H_2O$, 0·01 g.; $CaCO_3$, 3·5 g.; distilled water, 1000 ml.; (ii) peptone, 5 g.; glucose, 5 g.; distilled water, 1000 ml. Streptomyces isolates were grown in duplicate shaken flasks for 3 days at 24°. Sterilized Millipore filters (0·22 μ) were used to obtain sterile culture filtrates. Five ml. of each filtrate were applied to 13 mm. diam. paper discs by applying samples of 0·1 ml., then alternately air-drying the discs. The discs were placed on the surface of 0·5 % peptone

agar. The test fungus, *Glomerella cingulata*, was then sprayed on the agar surface. The assay plates were incubated at 24° for 3 days, when the zones of inhibition were observed. Control discs containing 5 ml. uninoculated media gave no inhibition zones.

Utilization of nutrients in agar. An agar medium was prepared containing: glucose, 2 g.; glutamic acid, 2 g.; KH_2PO_4, 5·3 g.; K_2HPO_4; 10·6 g.; agar, 25 g.; distilled water, 1000 ml. This medium, adjusted to pH 7·0, supported good growth of the test fungi and streptomycetes. To prepare an agar layer of uniform thickness, a top layer of 5 ml. was poured over a base layer of 5 ml. Four Streptomyces isolates were streaked on the agar surface. On the 1st, 2nd, 4th and 6th days, six agar discs, 7 mm. in diam., from the top layer of agar near the streptomycete colonies were removed and melted in 3 ml. glass-distilled water. Glucose was assayed enzymically (Keston, 1956) by the Glucostat reagent (Worthington Biochemical Corporation, Freehold, New Jersey), used according to the manufacturer's directions. Glutamic acid was assayed by melting three discs in 6 ml. glass-distilled water, using the ninhydrin method (Moore & Stein, 1954).

All experiments included several replicates and were done at least twice with similar results.

RESULTS

Inhibition characteristics of streptomycetes. Of the 20 streptomycetes, 18 caused inhibition zones (3 to 19 mm.) when *Glomerella cingulata* was used as the test organism (Table 1). Agar discs from the original inhibition zones of nine isolates caused new inhibition zones when transferred to fresh agar plates seeded with *G. cingulata* (Table 1). New inhibition zones were also produced by these isolates when paper discs placed beneath the original inhibition zones were transferred to fresh seeded agar. When agar discs from the original inhibition zones were transferred to fresh agar or to Petri dishes without agar, the test fungus failed to grow on discs of the same nine isolates. These results showed that diffusible inhibitory substances were produced by nine of the streptomycetes. Zone sizes for these isolates ranged from 12 to 19 mm., with a mean of 17 mm.

Neither agar discs from zones produced by the other nine antagonistic isolates nor paper discs from beneath such zones produced inhibition zones on fresh agar media seeded with *Glomerella cingulata*. Agar from zones produced by these isolates supported growth of the test fungus when discs were transferred to fresh agar or to Petri dishes without agar. The sizes of inhibition zones for these isolates ranged from 3 to 15 mm., with a mean of 8 mm. pH changes (from 6·9 to 8 ± 0·5) brought about in the agar by growth of the streptomycetes did not account for the inhibition since (i) *G. cingulata* grew well in that pH range, and (ii) similar inhibition zones occurred in media buffered with 0·1 M-phosphate, except where, in three instances, the buffer depressed growth of the streptomycetes.

Very similar results were obtained when *Mucor ramannianus* was used as the test fungus. All 20 Streptomyces isolates caused inhibition zones (7 to 19 mm.) on agar media. The same nine isolates produced inhibition zones, and the test fungus failed to grow on excised agar discs from these inhibition zones, even when a drop of 0·2 % glucose solution was added to the agar discs. Zone sizes for these isolates ranged from 14 to 19 mm., with a mean of 17 mm.

The other 11 isolates did not produce transferable inhibition zones and excised

agar discs from these original inhibition zones supported growth of the test fungus when a drop of glucose solution was added. The sizes of inhibition zones for these isolates ranged from 7 to 16 mm., with a mean of 11 mm.

Lysis characteristics of streptomycetes. Thirteen Streptomyces isolates produced zones of complete or partial lysis of *Glomerella cingulata* mycelium after 10 days incubation (Table 2). After 30 days, the sizes of these lytic zones had increased and five additional isolates had made small lytic zones.

Table 1. *Inhibition characteristics of streptomycetes against Glomerella cingulata in agar tests*

Streptomyces isolates	Original inhibition zone (mm.)*	Inhibition characteristics of agar from inhibition zones			
		New inhibition zones (mm.)†		Growth of test fungus‡	
		Agar	Paper discs	Agar	Paper discs
1 A	18	8	9	−	−
2 A	18	8	9	−	−
4 A	18	9	10	−	−
6 A	15	10	10	−	−
7 A	18	6	4	−	−
10 A	19	10	10	−	−
16 A	12	5	7	−	−
24 A	15	8	8	−	−
28 A	19	13	10	−	−
3 A	13	0	0	+	+
8 A	15	0	0	+	+
5 A	0	.	0	.	.
9 A	3	0	0	+	+
12 A	4	0	0	+	+
13 A	4	0	0	+	+
15 A	3	0	0	+	+
17 A	13	0	0	+	+
18 A	4	0	0	+	+
19 A	10	0	0	+	+
26 A	0	.	0	.	.

* Streptomycetes were streaked on the agar. After 3 days, conidia of *G. cingulata* were sprayed on the agar suface.

† Agar discs from inhibition zones or paper discs placed beneath the inhibition zones were transferred to fresh agar subsequently sprayed with conidia of *G. cingulata.*

‡ Agar discs from inhibition zones were transferred to unseeded agar or to Petri dishes without agar; −, = no growth of test fungus; +, = growth of test fungus.

Agar discs from lytic zones of these 18 streptomycetes were transferred to fresh test fungal cultures, but none made new lytic zones. The nine isolates which had earlier produced transferable inhibition zones again caused such zones on fresh, seeded peptone agar, and the other nine lytic isolates did not. Therefore, the lytic zones produced by the latter isolates must have been caused by factors other than antibiotics or diffusible lytic factors. At 30 days, the sizes of lytic zones containing antibiotics ranged from 10 to 15 mm., average 13 mm. The sizes of lytic zones without antibiotics ranged from 2 to 12 mm., average 7 mm.

Antibiotic production in liquid media. Sterile culture filtrates of 8 isolates (1 A, 2 A, 4 A, 6 A, 10 A, 16 A, 24 A and 28 A) contained antibiotic substances; these isolates all produced transferable inhibition zones on agar. Filtrates of eight other isolates (3 A,

5A, 7A, 8A, A9, 12A, 13A, 18A) contained no detectable inhibitory substances. Except for isolate 7A, these isolates did not produce transferable inhibition zones on agar plates. Isolate 7A grew poorly in the liquid media, which may account for its failure to produce antibiotics.

Table 2. *Lysis of Glomerella cingulata by streptomycetes in agar*

Streptomyces isolates	Original lytic zone (mm.)		Effect of transferred lytic zone*	
	10 days	30 days	Lytic zone	Inhibition zone (mm.)
I A	3	14	0	8
2 A	6	15	0	8
4 A	6	10	0	6
6 A	4	13	0	7
7 A	5	15	0	3
10 A	5	15	0	8
16 A	3	10	0	6
24 A	6	12	0	6
28 A	8	15	0	8
3 A	6	8	0	0
5 A	0	0	.	.
8 A	7	12	0	0
9 A	0	4	0	0
12 A	0	6	0	0
13 A	0	8	0	0
15 A	0	0	.	.
17 A	3	8	0	0
18 A	0	2	0	0
19 A	4	12	0	0
26 A	0	2	0	0

* Agar discs from lytic zones were transferred to agar containing mycelium or conidia of *G. cingulata*.

Fungal spore germination in streptomycete cultures. Four Streptomyces isolates were selected for further study. Isolates I A and 4A produced inhibitory substances; 3A and 8A did not. Conidia of *Glomerella cingulata*, which do not require exogenous nutrients for germination, were incubated in 3-day-old liquid cultures of these streptomycetes for 14 hr and germination was determined. In each of three experiments 150 to 180 spores were counted in each of 10 microscopic fields. Low mean levels of germination, 11 % and 18 %, occurred in cultures of isolates I A and 4A, respectively (Fig. 1) and were not significantly altered when potato broth was added at the time conidia were introduced. By contrast, high degrees of germination, 77 % and 72 %, occurred in cultures of 3A and 8A, respectively. When potato broth was added, 98 % of *G. cingulata* conidia germinated.

Effect of nutrient enrichment on inhibition zones. Isolates I A, 3A, 4A and 8A were streaked on agar media containing nine different concentrations (0·2 to 4·0 %) of peptone and glucose. After 3 days, conidia of *Mucor ramannianus*, a species requiring exogenous nutrients for germination, were sprayed on the agar surface. The size of inhibition zones produced by isolates 3A and 8A decreased with increasing nutrient concentration (Fig. 2). Agar discs from these inhibition zones produced no new inhibition zones when transferred to fresh seeded agar, and the test fungus grew when similar agar discs were transferred to unseeded agar. The sizes of inhibition zones produced by isolates I A and 4A did not decrease with nutrient concentration; agar
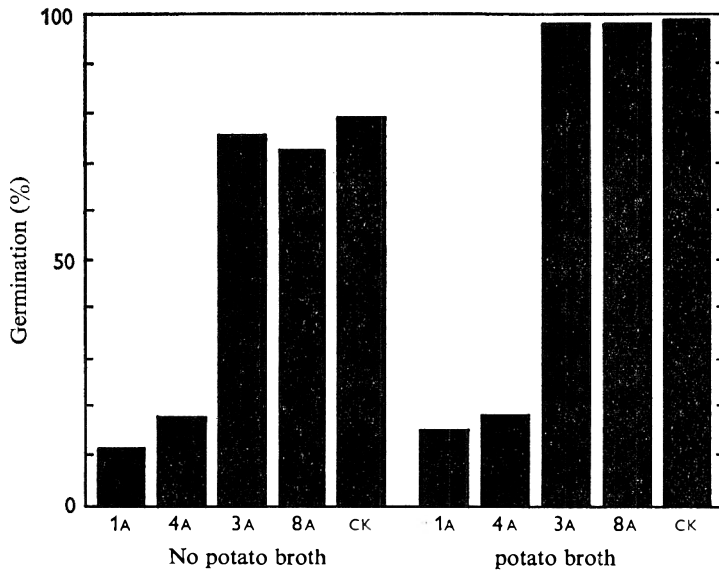
Fig. 1. Germination of *Glomerella cingulata* conidia in antibiotic (1 A and 4 A) and non-antibiotic (3 A and 8 A) streptomycete cultures.
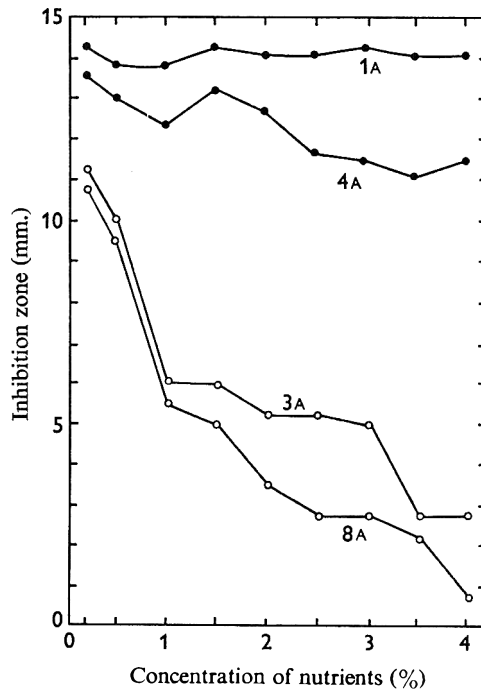

Fig. 2. Effect of concentration of glucose and peptone in agar on size of inhibition zones of *Mucor ramannianus* produced by antibiotic (1 A and 4 A) and non-antibiotic (3 A and 8 A) streptomycetes.

discs from these inhibition zones produced new inhibition zones when transferred to fresh seeded agar, and the test fungus did not grow when similar agar discs were transferred to unseeded agar.

Nutrient status of agar adjacent to streptomycete colonies. Glucose was rapidly lost from the agar within 7 mm. of streaks of isolates 1A, 3A, 4A and 8A: 60 to 70% was lost by the 2nd day, and 80 to 90% was lost by the 6th day (Fig. 3). Glutamic
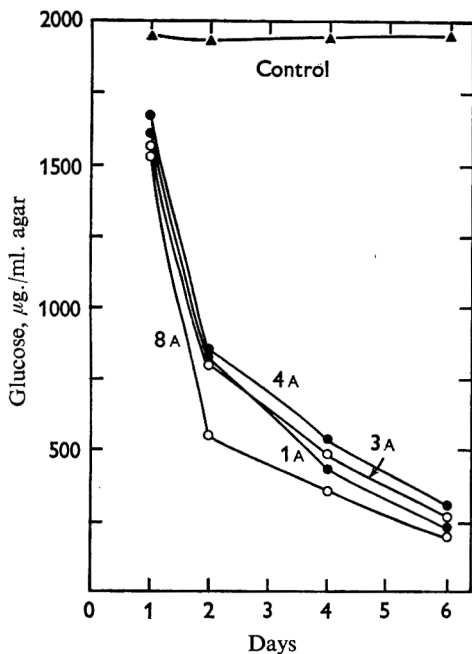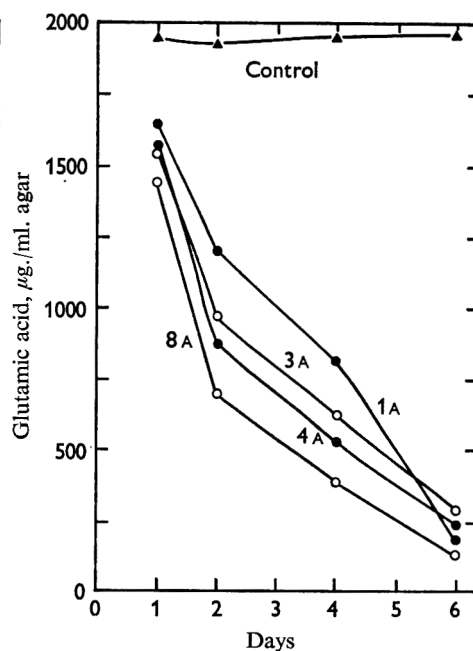

Fig. 3. Loss of glucose from agar in 7 mm. diam. discs immediately adjacent to antibiotic (1 A and 4 A) and non-antibiotic (3 A and 8 A) streptomycetes. Agar contained 0·2 % glucose and 0·2 % glutamic acid. Control was agar without streptomycetes.

Fig. 4. Loss of glutamic acid from agar in 7 mm. diam. discs immediately adjacent to antibiotic (1 A and 4 A) and non-antibiotic (3 A and 8 A) streptomycetes. Agar contained 0·2 % glucose and 0·2 % glutamic acid. Control was agar without streptomycetes.

acid was also rapidly utilized by the four streptomycetes: more than 75% was lost by the 6th day (Fig. 4). As with peptone-glucose agar, discs from the agar adjacent to isolate 1A and 4A, when transferred to fresh seeded agar, made new inhibition zones, but discs from the agar adjacent to isolate 3A or 8A did not make new inhibition zones.

Effect of glucose and glutamic acid concentration on germination of conidia of Mucor ramannianus. A low level (250 μg./ml.), approximating to the amount of each compound remaining in the agar after the 5th day, and a high level (2 mg./ml., the initial concentration) were prepared in the following mineral salt solution: $NaNO_3$, 2 g.; K_2HPO_4, 1 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; water, 1000 ml. Glucose and glutamic acid at 250 μg./ml. supported about 45% germination of *Mucor* spores, and 2 mg./ml. supported about 80% germination. The relatively lower amount of germination occurring at 250 μg./ml. may explain the occurrence of

the inhibition zones. Moreover, the depletion of nutrients would continue after the germination of the conidia, and the germ tubes would be exposed to an increasingly deprived environment. Observation of conidia of test fungi in inhibition zones showed that although some germination occurred, the germ tubes were unable to develop.

Inhibition zones produced in leached agar. To determine directly whether exhaustion of nutrients from agar can cause inhibition zones on agar, the following experiment was devised. A special Petri dish was prepared with the cover fitted with an inlet tube and the bottom with an outlet tube. The inlet tube was connected by vinyl tubing to a separatory funnel containing sterilized distilled water placed above the Petri dish. The outlet tube was similarly connected to a flask placed below the Petri dish. The entire system was autoclaved and maintained in a sterile condition. Twenty ml. of 0·5 % peptone glucose agar was poured into the Petri dish. A strip of agar about 7 mm. wide was removed, and replaced with a smaller amount of melted agar to seal the two semicircles of agar. The dish was tilted slightly and sterile distilled water was allowed to drip into one end of the groove and run out the other end into the sterile flask. The rate of water flow into the agar plate was adjusted to about 0·5 to 0·8 ml./ min. and continued for 7 days. At this time conidia of *Mucor ramannianus* or *Glomerella cingulata* were sprayed on the agar surface. Water was again allowed to run through the system for 3 more days, during which time clear inhibition zones about 10 mm. wide had developed on either side of the groove (Fig. 5). The spores of the test fungi in the inhibition zones had germinated poorly. When agar discs from these inhibition zones were transferred to fresh peptone agar, the conidia of both fungi germinated and developed mycelia. No contaminating microorganisms developed. Analysis of the agar in the zones showed that more than 90 % of the glucose and amino acid were lost during the 10-day period of the experiment. Therefore, leaching of nutrients from agar can make inhibition zones which duplicate in every way those produced by streptomycetes.

DISCUSSION

Eleven of the 20 inhibitory streptomycetes tested produced inhibition zones by a mechanism which did not involve antibiotics. Evidence for the lack of antibiotics may be summarized as follows: (i) Antibiotics could not be demonstrated in agar from inhibition zones or in paper discs placed beneath agar in inhibition zones; (ii) on excised agar from these inhibition zones, the nutrient-independent conidia of *Glomerella cingulata* germinated, and the nutrient-dependent conidia of *Mucor ramannianus* germinated when glucose was added; (iii) cultures of the same isolates in liquid media also failed to produce detectable antibiotics; (iv) conidia of *G. cingulata* germinated freely when incubated in cultures of these streptomycetes. By contrast, the nine other streptomycetes showed evidence for the presence of antibiotics in these tests.

Evidence that inhibition by non-antibiotic isolates was due to an induced deficiency of nutrients in the agar is: (i) sizes of inhibition zones produced by non-antibiotic streptomycetes decreased, whereas those produced by antibiotic streptomycetes were unchanged, when concentrations of nutrients in the medium were increased; (ii) glucose and glutamic acid were rapidly depleted to suboptimal levels in agar media adjacent to streptomycete colonies; (iii) agar, artificially deprived of nutrients by leaching, failed to support spore germination and mycelial growth. Such agar gave inhibition zones which resembled those produced by streptomycetes.

The failure of conidia of *Glomerella cingulata* to germinate in inhibition zones of non-antibiotic streptomycetes requires explanation, since it is nutrient-independent. Germination of *G. cingulata* conidia and some other nutrient-independent spores is known to be inhibited by artificial nutrient sinks, and their failure to germinate on soil has been explained on the basis of the nutrient sink provided by soil micro-organisms (Ko & Lockwood, 1967). The fact that agar in the inhibition zones produced by two antibiotic-producing streptomycetes was also rapidly deprived of nutrients raises a question as to the relative importance of antibiotics and nutrient depletion in production of inhibition zones by streptomycetes which produce antibiotics. Nutrient deprivation as an antagonistic mechanism in agar should also be evaluated for bacteria and fungi.

Although 18 Streptomyces isolates lysed fungal mycelia, in no case did agar discs from lytic zones make new lytic zones on fresh fungal cultures. However, new inhibition zones were made by those isolates shown to produce antibiotics. Therefore, the production of lytic zones can not be attributed to antibiotics or to any demonstrable diffusible lytic factors. Evidence that nutrient deprivation is involved in inducing autolysis of fungi in soil has been presented by Lloyd & Lockwood (1966). In view of the ability of four of the streptomycetes to effectively deplete agar of nutrients, it may be that lytic zones on agar result from nutrient deprivation.

Much research in plant pathology has dealt with determination of numbers of antagonistic micro-organisms in soils (Kaufman & Williams, 1965; Wood & Tveit, 1955). Attempts are often made to correlate populations of antagonists with incidence of disease in certain soil types or following some kind of soil treatment. Assessment of numbers of antagonists and degree of antagonism are done in agar tests. It is usually stated or implied that these data provide a measure of the numbers of organisms capable of producing antibiotics active against a given pathogenic fungus. The results of this research suggest that interpretation of these kinds of data on the basis of antibiotic production may be erroneous without further examination of the mechanism involved.

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EXPLANATION OF PLATE

Inhibition zone of *Glomerella cingulata* produced in leached agar.
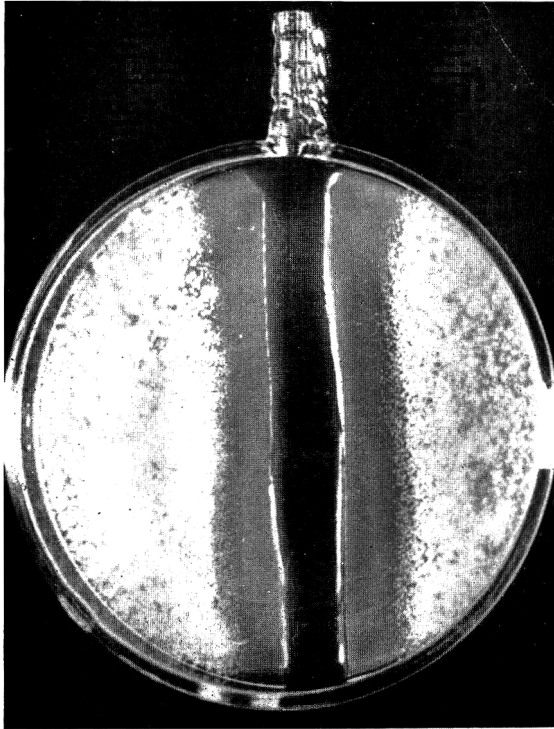
Plate 1

S. C. HSU AND J. L. LOCKWOOD

Self-inhibition of
Germination of Pycnidiospores of *Mycosphaerella ligulicola* in Relation to the Temperature of their Formation

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SUMMARY

At high concentrations, self-inhibition of germination of pycnidiospores of *Mycosphaerella ligulicola* increased with rise in temperature at which the spores were formed.

Spores formed at 15° (15° spores), which showed little self-inhibition at high concentrations, and 26° (26° spores), which showed marked self-inhibition at high concentrations, were used for experiments. Mixing 15° spores equally with 26° spores increased inhibition of the latter but the former were unaffected. Leachates from 15° and 26° cultures did not affect germination of 26° and 15° spores respectively, but germ tubes of 15° spores showed increased growth. Inhibition of 26° spores was overcome by washing ten times with deionized water and germ tube growth from washed spores was increased in culture leachates. Diffusates collected from dense suspensions of 26° spores did not affect germination. Diffusates from 15° spores prevented germination of 26° spores but not 15° spores where growth of germ tubes was increased. The inhibitory substance from 15° spores was not readily volatile and not affected by high temperature in solution. Volatile inhibitors were not detected from either 15° or 26° spores. Pfeffer 1 % glucose solution overcame self-inhibition of 26° spores but 1 % glucose or Pfeffer solution alone were ineffective.

INTRODUCTION

A rise in incubation temperature of the chrysanthemum ray blight pathogen (*Mycosphaerella ligulicola*, Baker, Dimock & Davis) between 12 and 27° resulted in an increase in pycnidiospore number which was associated with a decrease in size (Blakeman & Hadley, 1968). Further work (Blakeman & Fraser, 1969) showed that at high concentrations spores formed at 26° showed greater self-inhibition of germination when compared with spores formed at 15°. There appear to be no reports of a connexion between incubation temperature of cultures and self-inhibition of the spores for other fungi. The ability of spores, formed over a range of temperatures, to germinate at different concentrations was examined further and the nature of differences in self-inhibition between spores formed at two selected temperatures was investigated.

METHODS

Spores of *Mycosphaerella ligulicola* were collected by adding 6 ml. of deionized water to the surface of Petri dish potato glucose agar cultures and gently shaking on a rotary shaker for 30 min. The filtrate (referred to as a culture leachate) was removed

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by passage through a membrane filter and the deposit of spores which remained on the membrane was normally washed by passing through two or more batches of 20 ml. deionized water. In some cases spores were further washed with up to twenty lots of 20 ml. water. The liquid (referred to as a spore leachate) was collected and concentrated after passage through the membrane. The washed spores were removed by cutting the membrane into four strips and shaking in deionized water in a universal bottle. The concentration of the suspension was measured by haemocytometer and diluted as required. Tests were made to determine the effect on germination of culture leachates and spore leachates. Also dense suspensions of previously washed spores were placed in bijou bottles and shaken gently for 12 hr (spores do not germinate under such conditions). After passage through a membrane filter to separate the spores, the filtrate (referred to as a spore diffusate) was tested for its effect on germination. Germination tests were made on Cellophane (British Cellophane Co., PT 300) discs (1·5 cm. diam.), placed on a slide and covered with a coverslip supported on a glass ring which was sealed with petroleum jelly to the slide. All slides were incubated in Petri dish moist chambers for 18, 24 or 48 hr, after which each portion of Cellophane was transferred to a clean slide, mounted in a drop of acid fuchsin stain and covered with a coverslip which was sealed to the slide with nail varnish. Assessment of germination (from samples of at least 100 spores) and length of the longest germ tube from each spore (from samples of 50 germinated spores) were made on the preserved material. Standard deviations for percentage spore germination were calculated using the arcsin transformation and a 't' test was applied to data for germ tube lengths.

The method used to collect volatile materials from spores was as follows. Thirty ml. of dense spore suspension in water was placed in each of four interconnected Dreschler bottles. Filtered air was bubbled through for 6 hr and condensed in a U tube immersed in, or placed 1 to 2 cm. above, liquid nitrogen in a Dewar flask. On transference of the tube, after immersion in the liquid nitrogen, to room temperature, condensed oxygen was given off violently causing loss of any volatiles which might have condensed in it. There was no condensation of oxygen when the tube was placed above the nitrogen where the temperature was above the boiling point of oxygen. Alternatively a mixture of solid carbon dioxide in ethanol was used for the condensation. After collection of volatiles the U tube was sealed with rubber stoppers and stored in a deep freeze. The melted contents of the U tube were subsequently tested for their effect on germination of spores of *Mycosphaerella ligulicola.*

RESULTS

Rise in incubation temperature, from 12 to 27°, of cultures of *Mycosphaerella ligulicola* increased self-inhibition of the spores when tested at concentrations ranging from 0.75×10^6 to 30×10^6/ml. at 20° (Fig. 1).

Mixing equal numbers of spores formed at 26° with those formed at 15° did not prevent germination of the latter but caused increased inhibition in the former. Suspending 26° spores in leachates from 15° cultures did not overcome self-inhibition. Conversely there was no decrease in germination on suspending 15° spores in leachates from 26° cultures (Fig. 2) and the germ tubes, after 18 hr incubation, were nearly three times as long as those of control spores germinated in water (Table 1). The number of germ tubes arising from each 15° spore was nearly twice that from each 26° spore

regardless of differences in treatments used. No lateral branches arose from germ tubes of 26° spores after 18 hr but a few lateral branches had developed from 15° spores germinated in water and over three times as many from spores suspended in leachates from 26° cultures.

The effect of prolonged washing on germination of 26° spores was investigated. Spores washed ten times showed less self-inhibition when compared with unwashed spores. Resuspending washed spores in a culture leachate resulted in 90 to 100%
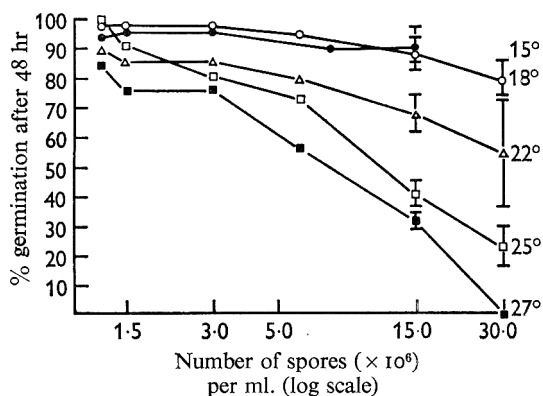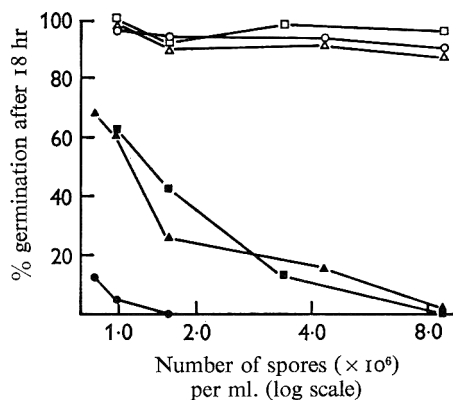

Fig. 1.   Fig. 2.

Fig. 1. Effect of spore concentration on germination of spores of *Mycosphaerella ligulicola* formed at different temperatures. Standard deviations are given for the higher densities.

Fig. 2. Effect of mixed spore suspensions of *Mycosphaerella ligulicola* on germination of 15° (O) and 26° (●) spores; culture leachates on germination of 15° (△) and 26° (▲) spores; comparison with germination of 15° (□) and 26° (■) spores in deionized water.

Table 1. *Effect of culture leachates on germ tube growth of*
Mycosphaerella ligulicola

	Spores washed and suspended in			
	15° culture leachate	26° culture leachate	Deionized water	
Test spores	26°	15°	26°	15°
Germ tubes (mean no. per spore)	1·2	2·3	1·2	2·2
Lateral branches (mean no. per spore)	0	1·7†	0	0·5
Mean length (μ) of longest germ tube per spore	7·6*	95·2†	10·8	34·0

When compared with unwashed controls: $*P = 0·01$, $†P = 0·001$.

Measurements made from samples of 50 spores after 18 hr incubation at a concentration of $0·85 \times 10^6$ per ml.

germination over the range of spore concentrations tested (Fig. 3). Germ tubes after 48 hr incubation were three times as long after the washing of spores and about fifteen times as long when washed spores were suspended in a culture leachate (Table 2). In the latter the number of germ tubes was nearly twice that of washed or unwashed spores in water. Unwashed spores had no lateral branches after 42 hr but washed spores, suspended in water or culture leachate, had approximately one lateral branch developing from the primary germ tubes from each spore.
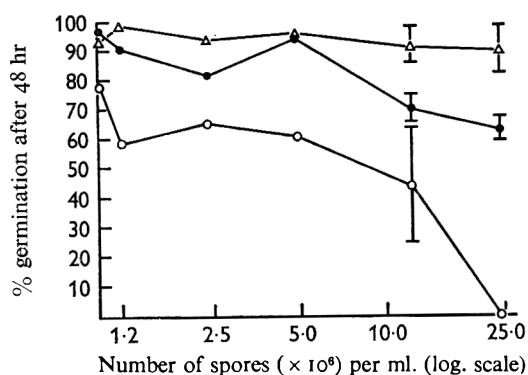
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Fig. 3. Germination of unwashed 26° spores (O) of *Mycosphaerella ligulicola* and ten times washed spores suspended in water (●) and in a culture leachate (△) at different concentrations. Standard deviations are given for the higher concentrations.
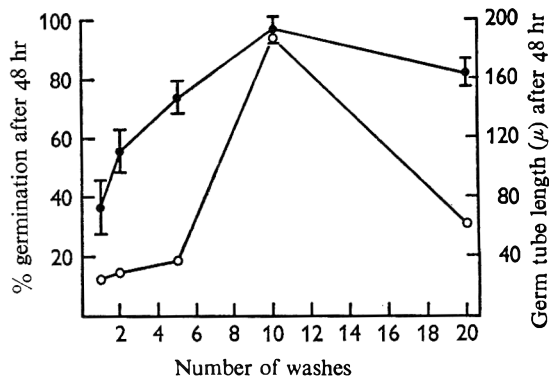
Fig. 4. Effect of washing on germination (●) and germ tube growth (O) of 26° spores of *Mycosphaerella ligulicola*.

Table 2. *Effect on germ tube growth of washing and resuspending 26° spores of Mycosphaerella ligulicola in a culture leachate*

	Spores washed ten times	Spores washed ten times and resuspended in a culture leachate	Unwashed spores
Germ tubes (mean no. per spore)	1·5	2·8	1·5
Lateral branches (mean no. per spore)	0·8*	1·3*	0
Mean length (μ) of longest germ tube per spore	27·6*	140·0*	9·2

* $P = 0·001$ when compared with unwashed controls.

Measurements made from samples of 50 spores after 48 hr incubation at a concentration of $0·7 \times 10^6$ per ml.

Further studies were made by washing 26° spores once, twice, five, ten and twenty times and testing germination at 30×10^6, $3·4 \times 10^6$ and $0·65 \times 10^6$ spores/ml. Results (Fig. 4) are given for a concentration of $3·4 \times 10^6$. The trends shown at the other concentrations were similar though of a different order. With rise in the number of washes up to ten, germination and growth of germ tubes increased. Suspending spores washed twenty times in a culture leachate increased germination and germ tube growth only at the lowest concentration tested. Suspending washed spores in spore leachates, obtained from concentrating the collected material from twenty washes (600 ml.) to a volume of 3 ml. in a rotary evaporator, significantly decreased germination only at the highest concentration tested. At a concentration of $3·4 \times 10^6$ spores/ml. growth of germ tubes was diminished being similar to unwashed control spores, but at $0·65 \times 10^6$/ml. germ tubes were shorter than unwashed controls (Table 3).

Diffusates from 26° spores (at concentrations of 36×10^6 and 360×10^6 spores/ml.) were shown to increase germination and growth of germ tubes of 26° spores (Table 4), the amount depending on the concentration of the diffusate (Table 5). Diffusate from 26° spores had no effect on germination of 15° spores (Table 4), but, as with 26° test

Table 3. *Effect of culture leachates and concentrated spore leachates on germination of washed 26° spores of Mycosphaerella ligulicola*

Spores washed 20 times and germinated in

	Culture leachates			Spore leachates			Deionized water			Unwashed spores		
Concentration of test spores ($\times 10^6$)	30	3·4	0·65	30	3·4	0·65	30	3·4	0·65	30	3·4	0·65
% germination (with standard deviation)	44±6·5	80±7·5	94±4·5	26±6·5	68±9·5	88±9·0	55±5·0	82±5·0	74±4·0	36±6·5	37±8·5	64±10·5
Germ tube length (μ)	—	63·3†	86·7†	—	28·7	33·2*	—	62·0†	43·6	—	24·5	46·8

When compared with unwashed controls: * $P = 0.05$, † $P = 0.001$. Measurements made after incubation for 48 hr.

Table 4. *Effect of spore diffusates on germination and germ tube growth of Mycosphaerella ligulicola*

Test spores (concentration 1.4×10^6)

	15° spores			26° spores		
Treatment	15 °spore diffusate	26° spore diffusate	Deionized water	15 °spore diffusate	26° spore diffusate	Deionized water
% germination after 24 hr	81±5·5	99±4·0	99±2·6	0	94±7·5	74±9·0
Germ tube length (μ) after 24 hr	104·8*	43·6*	26·4	0	25·2*	13·6

* $P = 0.001$ when compared with spores germinated in deionized water.
15° and 26° spore diffusates collected from suspensions containing 58×10^6 and 36×10^6 spores/ml. respectively.

spores, the growth of germ tubes was related to the concentration of the diffusate (Table 5).

Diffusates from 15° spores (at 46×10^6 and 58×10^6 spores/ml.) caused a fourfold increase in germ tube growth of 15° test spores when compared with spores germinated in water. This was accompanied by a small decrease in germination possibly due to rapidly spreading hyphae inhibiting spores which had not germinated. On the other hand, diffusate from 15° spores caused complete inhibition of germination of 26° test spores (Table 4). After 48 hr in contact with the diffusate a sample of the spores was removed, washed and transferred to potato glucose agar when only 6 % of the spores

Table 5. *Influence of dilution of diffusate from 26° spores (360×10^6/ml.) on germination and germ tube growth of 15 and 26° spores of Mycosphaerella ligulicola*

Dilution of spore diffusate	Undiluted		1/2		1/3	
Test spores formed at 15 or 26° (Concentration 1.4×10^6)	15	26	15	26	15	26
% germination (with standard deviation) after 24 hr	99 ± 3.0	94 ± 7.5	98 ± 3.5	85 ± 6.5	98 ± 4.0	81 ± 8.5
Germ-tube length (μ) after 24 hr	$92.4*$	$42.8*$	—	—	—	—

Dilution of spore diffusate	1/4		1/8		Deionized water	
Test spores formed at 15 or 26° (Concentration 1.4×10^6)	15	26	15	26	15	26
% germination (with standard deviation) after 24 hr	100 ± 0	88 ± 7.0	99 ± 1.0	79 ± 6.0	98 ± 5.0	79 ± 6.0
Germ-tube length (μ) after 24 hr	$28.0*$	$17.6*$	—	—	12.8	6.8

* $P = 0.001$ when compared with spores germinated in deionized water.

Table 6. *Influence of dilution of diffusates from 15° spores (58×10^6/ml.) on germination and germ tube growth of 26° spores of Mycosphaerella ligulicola*

Dilution of spore diffusate	Un-diluted	1/2	1/3	1/4	1/8	Deionized water
% germination (with standard deviation) after 24 hr	0	24 ± 8.5	45 ± 8.0	50 ± 3.5	67 ± 12.5	92 ± 4.0
Germ-tube length (μ) after 24 hr	0	$10.0*$	$11.6*$	$17.2*$	$11.6*$	28.4

* $P = 0.001$ when compared with spores germinated in deionized water.
Concentration of test spores 1.4×10^6/ml.

grew. Dilution of the diffusate caused a steady increase in germination. Growth of germ tubes of those spores able to germinate was decreased in all dilutions tested as compared with water controls (Table 6).

Investigations were made as to whether the inhibitory effect of diffusates from 15° spores was affected by temperature or was volatile. Samples (1 ml.) of diffusate were placed in bijou bottles, both capped and uncapped, and maintained at temperatures of 2, 20, 37 and 95° for 18 hr. Afterwards the solutions were used at 20° to

determine whether they would support germination. There was a complete inhibition of spore germination using diffusates maintained at temperatures of 2, 20 and 37° in both capped and uncapped bottles, and at 95° in capped bottles only. The diffusate in the uncapped bottle at 95°, which had dried leaving a yellowish deposit, was dissolved in 1 ml. deionized water and added to 26° test spores where it enhanced germination (after 48 hr) and increased growth of germ tubes by ten times (Table 7).

Table 7. *Germination of 26° spores of Mycosphaerella ligulicola at 20° in diffusate from 15° spores previously maintained for 18 hr at different temperatures in capped (c) or uncapped (uc) bottles*

		Temperature (°)							Deionized water control
		2		20		37		95	
		c	uc	c	uc	c	uc	c	uc	
% germination	24 hr	0	0	0	0	0	0	48 ± 11.5	0	53 ± 9.0
	48 hr	0	0	0	0	0	0	100 ± 0.5	0	69 ± 6.5
Germ-tube length (μ) 48 hr		—	—	—	—	—	—	204.0*	—	21.2

* $P = 0.001$ when compared with spores germinated in deionized water.
Concentration of test spores 1.4×10^6/ml.

Table 8. *Germination of spores of Mycosphaerella ligulicola after 24 hr at different concentrations in water and nutrient solutions*

Spore concentration/ml.	Water	1 % glucose	Pfeffer soln	Pfeffer soln + glucose (1 %)
1×10^6	73 ± 8.0*	53 ± 15.0	79 ± 4.0	84 ± 6.5
15×10^6	13 ± 5.5	3 ± 5.5	41 ± 7.0	94 ± 7.5
30×10^6	0 ± 1.5	7 ± 6.0	3 ± 6.0	99 ± 2.5

* % germination.

Table 9. *Germ tube length 48 hr after incubation of spores of Mycosphaerella ligulicola at different concentrations in water and nutrient solutions*

Spore concentration/ml.	Water	1 % glucose	Pfeffer soln	Pfeffer soln + glucose (1 %)
1×10^6	30.8‡	74.0†	40.8*	290.4†
15×10^6	12.8	15.6	58.8†	260.6†
30×10^6	3.3	11.6†	20.8†	135.3†

When compared with spores germinated in deionized water: * $P = 0.05$, † $P = 0.001$.
‡ Germ tube length μ.

Addition of condensed volatiles obtained from either 15° or 26° spores and collected in a U tube mounted a few cm. above or below the surface of liquid nitrogen or in a mixture of solid carbon dioxide + ethanol caused no change in germination or growth of germ tubes from that of control spores suspended in deionized water.

Tests on the germination of 26° spores at different concentrations in nutrient solutions demonstrated (Table 8) that addition of 1 % glucose or Pfeffer solution ($Ca(NO_3)_2$, 0.8 g.; $MgSO_4$, 0.2 g.; KNO_3, 0.2 g.; KCl, 0.1 g.; K_2HPO_4, 0.2 g.; trace-element supplement; made to 1 l. with deionized water) did not overcome the inhibition of germination shown at high spore concentrations. On the other hand provision

of Pfeffer solution + 1 % glucose completely overcame the inhibition of germination even at the highest concentration tested (30×10^6 spores/ml.). The provision of 1 % glucose or Pfeffer solution, separately, in most cases caused some increase in the growth of germ tubes of those spores able to germinate, but much greater increases occurred with Pfeffer solution + 1 % glucose (Table 9).

DISCUSSION

Self-inhibition of germination of spores of *Mycosphaerella ligulicola* has been shown to depend on the incubation temperature of the parent culture. Mixing 26° spores (which show marked self-inhibition related to suspension concentration) equally with 15° spores (in which self-inhibition is much less evident) showed that the inhibitory condition associated with the former did not affect germination of the latter. But the presence of 15° spores in the mixture resulted in a greater inhibition of 26° spores. This suggests that conditions which inhibit germination of 26° spores are associated with both 15° and 26° spores. Either 15° spores are insensitive to their effects or they possess a mechanism to overcome them.

The possibility that the inhibitory condition originated from the parent culture was examined. Leachates from cultures did not decrease the proportion of germinating 15° or 26° spores even at low concentrations but growth of germ tubes of 15° spores was substantially increased. This indicates that the inhibitory condition may originate from the spores themselves. Attempts were made to remove inhibition by washing the spores. A large increase in the proportion of germinating spores, coupled with the development of longer germ tubes (particularly at high concentrations) occurred with ten times washed 26° spores. Washed 26° spores were able to respond to a culture leachate in the same way as unwashed 15° spores, germ tubes showing a further increase in length. More prolonged washing (20 times) resulted in a slightly decreased germination and shorter germ tubes, due possibly to a decrease in endogenous reserves through leaching. The effect of washing might be to remove a germination inhibitor but application of a concentrated leachate obtained from twenty collected spore washings did not inhibit washed spores at the lower concentrations tested. Only at the highest concentration was there some restriction of germination. The length of germ tubes, however, was decreased at all concentrations. It is possible that washing of spores may have resulted in loss of a substrate required for the formation of an inhibitor.

Diffusates collected from dense suspensions of 26° spores did not affect germination but caused enhanced growth of germ tubes of both 15° and 26° spores. Self-inhibition of 26° spores would seem therefore not to be due to water-soluble inhibitors diffusing from spores unless these inhibitors were also rapidly labile. Alternatively a sufficient concentration of inhibitors may not have been attained. Only when tested on 15° spores did diffusates from 15° spores have the same effect as a 26° spore diffusate. The observation that diffusates from 15° spores completely inhibit germination of 26° spores may explain the increased inhibition of the latter when mixed with 15° spores. It also shows the greater sensitivity of 26° spores to inhibitory substances. The active material from 15° spores is not readily volatile and not affected by prolonged exposure to a high temperature in solution. From these results it is clear that the inhibitor causing self-inhibition of 26° spores is different in nature from that produced by 15° spores.

The presence of volatile inhibitors of spore germination was not detected from suspensions of either 26° or 15° spores, though they have been obtained from the mycelium of *Fusarium oxysporum* (Robinson & Park, 1966) and from rust uredospores (Allen, 1955.)

The observation that inhibition of germination of 26° spores at high concentrations can be overcome by provision of a defined nutrient solution might indicate that lack of nutrients was the cause of the inhibition. Nutrients are more likely, however, to overcome an inhibitory condition associated with the spores since germination of spores at high concentrations can take place after prolonged washing of the spores, during which nutrient levels within the spores are likely to be decreased through leaching. The virtual absence of self-inhibition of germination of 15° spores may be attributed to the higher levels of nutrient reserves contained in the larger spores which may, on germination, overcome the action of inhibitors which have been shown to be associated with these spores. Occasional larger spores present in dense suspensions of 26° spores were invariably observed to germinate more readily.

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Steady State Levels of Dehydrogenases and α- and β-Glucosidases in *Klebsiella aerogenes*

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SUMMARY

Klebsiella (*Aerobacter*) *aerogenes* (NCIB 418, *Bacterium aerogenes* no. 240) was grown at dilution rates between 0·1 and 1·0 hr^{-1} in a variety of nutrient-limited chemostats and the activities of dehydrogenases, particularly glucose (R_G), fructose (R_F), sucrose (R_S), maltose (R_M) and gluconate (R_{GN}) dehydrogenases, were determined in intact organisms. Their activities varied from system to system, but, with a few exceptions, were largely independent of the dilution rate. R_G was generally high when sugars provided the carbon for growth but when intermediates of the tricarboxylic acid cycle were used it was often low. R_{GN} behaved like R_G but R_F, R_S, and R_M were more variable. In general the activities were higher in C-limitation than in other nutrient-limited conditions.

Specific activities of α- and β-glucosidase were also determined in some of the systems. Organisms grown in a maltose-limited system had the highest α-glucosidase activity but 40% of this level was observed in those from a cellobiose-limited system although cellobiose is a β-glucoside. Glucose, fructose, sucrose, lactose or disaccharides containing an α 1→6 linkage as carbon sources induced little activity. As expected, growth in cellobiose-limited conditions led to high β-glucosidase activity but melibiose-grown cells were about 50% as active. Replacing NH_4^+, the usual N source in the medium, by NO_2^- in a glucose-limited system increased the β-glucosidase activity five- to sixfold, while NO_3^- led to a tenfold decrease. α-Glucosidase was less affected.

INTRODUCTION

Although their need has been pointed out (Pirt, 1967; Málek, 1969) relatively few studies have appeared on the activities of enzymes in bacteria growing in the steady state of continuous culture. Those which have, however, indicate the usefulness of the approach (see for example Tempest & Herbert, 1965; Wright & Lockhart, 1965; Hamlin, Ng & Dawes, 1967; Clarke, Houldsworth & Lilly, 1968), since if the chemostat technique is used the effect of changes in the dilution (growth) rate and the nature of the rate-limiting nutrient in the medium can be assessed. In this paper the effect of these variables on the steady state specific activities of some dehydrogenase systems in *Klebsiella* (*Aerobacter*) *aerogenes* (NCIB 418, *Bacterium aerogenes* strain no. 240), particularly those acting on glucose, gluconate, fructose, maltose and sucrose is assessed. The α- and β-glucosidase activity of the cells is also examined although in less detail.

METHODS

The continuous culture apparatus and the general experimental techniques have been described elsewhere (Dean & Rogers, 1967). The standard salts glucose medium contained (g./l.): $FeSO_4.7H_2O$, 0.2×10^{-3}; $MgSO.7H_2O$, 0.039; $(NH_4)_2SO_4$, 0.96; KH_2PO_4, 1.14; $Na_2HPO_4.12H_2O$, 6.13; glucose, 19.2; pH 7.1. For chemostat operation the concentration of the rate-limiting nutrient was reduced to a level which gave a steady state cell mass in the culture vessel of 0.12 mg. dry weight/ml. over as wide a range of dilution rates as possible. In C-limited conditions this was 300 mg./l. when sugars and related substances were the carbon sources and 1 g./l. when intermediates of the tricarboxylic acid cycle and related substances were used. NH_4^+, K^+, Mg^{2+}, SO_4^{2-} and PO_4^{3-}-limited systems contained respectively 27.3, 2.0, 0.35, 1.4 and 10.0 mg. ions/l. Cultures were grown at 40° and air at 1 l./min. was passed through them.

For the enzyme assays organisms were separated from the growth medium by centrifugation, washed with NaCl (0.85 g./l.) and resuspended in phosphate buffer (KH_2PO_4, 2.96; $Na_2HPO_4.12H_2O$, 16 g./l.; pH 7.1) at a concentration of 0.5 to 1.2 mg. dry weight/ml. (Using concentrations within this range in the assays as described below gave enzyme activities which were independent of the cell mass in the reaction mixtures.) When necessary the organisms were next disrupted in various ways: (i) ultrasonic treatment for 1 to 4 min. ice-cold; (ii) adding 1% w/v of benzene and shaking for 1 to 5 min. at 30°; (iii) incubating at 40° for 24 hr with 0.01% (w/v) cetyltrimethylammonium bromide or 0.1% (w/v) sodium lauryl sulphate; (iv) using the lysozyme-sucrose-EDTA method of Birdsell & Cota-Robles (1967). The fragments and the medium in which the organisms had been disrupted were assayed together.

Dehydrogenase activities were measured by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in the presence of the appropriate substrate. One ml. of suspension was added to five tubes containing 1 ml. carbon substrate under investigation and 2 ml. phosphate buffer (pH 7.1). After 10 min. incubation in a 40° thermostat, 1 ml. freshly prepared TTC solution (1 mg./ml.) was added to each tube to start the reaction (the pre-incubation period gave maximum activity); at intervals tubes were removed from the water bath and 10 ml. acetone added to stop the reaction and extract the formazan. The tubes were then stoppered and after 24 hr at room temperature (when all the debris had precipitated) the extinction was measured at 485 mμ. Since disturbance of the surface of the reaction mixture leads to changes in the reaction rate (Fred & Knight, 1949) the use of separate tubes was preferable to withdrawing samples from a bulk reaction mixture. Plots of extinction against time were usually linear over the first 30 min. and activities were always calculated from initial reaction rates, the amount of TTC reduced being obtained from a calibration curve relating it to extinction. This was obtained by reducing TTC solutions of known concentration with an excess of sodium hydrosulphite in the presence in turn of the various carbon sources used in the chemostat experiments. The amount of TTC reduced was independent of the carbon source and a linear relation existed between it and the extinction in the concentration range 0.015 to 1.0 μM. A blank in which 1 ml. phosphate buffer replaced the carbon source in the assay was run in parallel and its value (which was taken as a measure of the endogenous enzyme activity) was subtracted from the observed activity to give the exogenous dehydrogenase activity.

This is expressed as specific activity, i.e. mμ moles TTC reduced/mg. dry weight organisms/min.

Activities of α- and β-glucosidase were determined by adding 5 ml. of *p*-nitrophenol (α or β)-D-glucopyranoside to 20 ml. suspension shaken at 40°. Five ml. samples were withdrawn at intervals, 2 ml. 0·1 N-NaOH added to stop the reaction, and the extinction determined at 400 to 420 mμ with reference to a blank withdrawn at zero time. Initial rates were again determined and glucosidase specific activity is defined as mμ moles *p*-nitrophenol liberated/mg. dry weight organisms/min.

<center>RESULTS</center>

Effect of pH value, temperature, air flow rate and cell disruption

Changing the pH value of the medium or the temperature of growth had little effect on the steady state glucose dehydrogenase specific activities (R_G) of organisms grown in glucose or NH_4^+-limited chemostats until the conditions were so adverse as to retard growth, viz. pH $<$ 5, $>$ 8; temperature $>$ 40°. R_G also remained constant at air flow rates through the medium between 0 and 1 l./min. The polysaccharide content of the organisms increased at low temperatures and the yield decreased at air flow rates below 0·8 l./min. Accordingly, a temperature of 40° and an air flow rate of 1 l./min. were chosen as standard conditions since any slight uncontrolled variations would have a negligible effect.

Disruption of the organisms led to lower glucose dehydrogenase activities. The cetyltrimethylammonium bromide or sodium lauryl sulphate methods resulted in complete inactivation while ultrasonic treatment or treatment with benzene or lysozyme and EDTA led to a 40 to 50 % reduction. Adding FAD (4×10^{-4}M), NADP (3×10^{-5}M) or ATP (4×10^{-5}M) to the assay mixtures had no effect. Glucosidase activities were also decreased by ultrasonic treatment; other methods of disruption were not tested. Since optimum levels of activity were desired, intact organisms were used throughout the experiments which follow.

Dehydrogenase activities in carbon-limited systems

Steady state glucose (R_G), fructose (R_F), maltose (R_M) and sucrose (R_S) dehydrogenase specific activities were determined in organisms growing in a wide variety of carbon-limited chemostats at dilution (growth) rates from 0·1 to 1·0 hr^{-1}. Figure 1a shows that in the glucose-limited system R_G, R_F, R_M and R_S were equal and remained at a constant level at all except very low dilution rates. In all the other systems R_G, R_F, R_M and R_S differed depending on the carbon source in the medium, but since, with a few exceptions which will be pointed out presently, a similar independence of dilution rate occurred throughout, the constant levels only need be reported. These are listed in Table 1, which shows that when sugars and related substances replaced glucose as the carbon source in the medium, R_G remained high, only dropping below 80 % of the control (glucose-limited) level in the D-arabinose system (Table 1a). On the other hand, R_G varied considerably and was often low when intermediates of the tricarboxylic acid cycle and related substances were the carbon sources (Table 1b). Moreover in this latter group of experiments a marked dependence of activity on dilution rate occured three times. In the malate-limited system this took the form of a linear decrease in R_G as the dilution rate increased, while in the succinate- and

citrate-limited systems maximum values occurred at dilution rates of 0·2 and 0·4 hr^{-1} respectively. (The values given in Table 1b for these systems are maximum values.) Organisms grown in gluconate and glycerol-limited systems had appreciable levels of R_G (Table 1c). These substances are listed separately since the behaviour observed when they were used in NH_4^+-limited systems was atypical. This is dealt with in the next section.
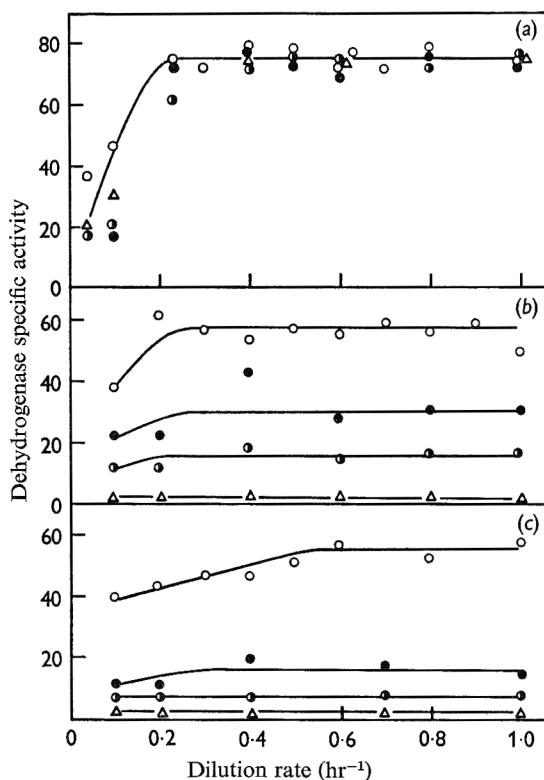

Fig. 1. Steady state glucose (○), fructose (●), sucrose (◗) and maltose (△) dehydrogenase specific activities when glucose was the carbon source in the growth medium. (a) C-, (b) K$^+$- and (c) NH_4^+-limited chemostats.

The exact correspondence between R_G, R_F, R_M and R_S in the glucose-limited system was unique. The activities of other dehydrogenases in glucose-grown organisms varied considerably. For example, the specific activities of those acting on D-mannitol, lactose, L-arabinose, gluconate, L-glutamate, glycerol, pyruvate and lactate amounted to 75, 23, 19, 17, 14, 10, 7 and 3 % respectively of R_G. Moreover, in all the other systems listed in Table 1 in which significant levels of activity were detectable, R_G, R_F, R_M and R_S, rather than being equal as in the glucose-limited system, usually fell in the order $R_G > R_F > R_M$ or R_S. R_S, however, was high in the fructose, maltose and raffinose-limited systems and in conditions in which fructose, maltose or sucrose had acted both as the carbon source in the growth medium and as substrate in the enzyme test, the level of activity of the relevant dehydrogenase (i.e. R_F in fructose-grown, R_M in maltose-grown organisms, etc.) increased considerably. This proved to be of general occurrence when the activities of other dehydrogenases such as those

acting on D-mannitol, L-arabinose, lactose, malate, glycerol, pyruvate, lactate and L-glutamate were examined. Gluconate dehydrogenase, on the other hand, behaved more like glucose dehydrogenase. In the gluconate-limited system the gluconate dehydrogenase specific activity (R_{GN}) of the organisms was 24·6 units. Here optimum activity might be expected since the carbon substrate performs the dual role just described. Like R_G, however, similar levels of activity occurred in many other systems and indeed in all the conditions in which R_G was high, except the fructose-limited system, R_{GN} never varied by more than about 40%, and when R_G was low, R_{GN} was also low.

Table 1. *Dehydrogenase activities in carbon-limited chemostats*

Carbon source in medium	Relative activity*				
	R_G	R_F	R_S	R_M	R_{GN}
(a) Glucose	1·00	1·00	1·00	1·00	0·22
Fructose	0·96	0·90	0·87	0·02	0·02
Sucrose	1·14	0·59	1·12	0·17	0·25
Maltose	0·87	0·51	0·70	0·87	0·21
Xylose	1·20	0·71	0·04	0·29	0·28
D-mannitol	1·02	0·80	0·21	0·32	0·35
Inositol	1·03	0·71	0·10	0·30	0·31
Lactose	0·80	0·30	0·03	0·04	0·25
Melibiose	1·00	0·40	0·10	0·39	0·31
Cellobiose	1·01	0·41	0·11	0·04	0·20
Raffinose	0·86	0·50	0·71	0·29	0·24
L-arabinose	1·00	0·51	0·03	0·03	0·30
D-arabinose	0·37	0·20	0	0	0·21
(b) Fumarate	0·81	0·52	0·16	0·24	0·25
DL-glycerate	0·64	0·38	0·03	0·20	0·21
Malate†	0·57	0·33	0·17	0·09	0·20
cis-Aconitate	0·36	0·10	0	0	0·08
Citrate†	0·11	0·05	0·10	0·06	0·07
Lactate	0·11	0	0	0	0·06
L-glutamate	0·06	0·05	0	0	0·08
Succinate†	1·00	0·72	0·35	0·02	0·37
Pyruvate	0	0	0	0	0·12
Malonate	0	0	0	0	0·03
Acetate	0	0	0	0	0·12
(c) Gluconate	0·53	0·20	0·18	0	0·34
Glycerol	0·67	0·64	0·17	0·32	0·23

* The glucose dehydrogenase specific activity of intact organisms grown in glucose-limited conditions (i.e. 75·0 mμ moles TTC reduced/mg. dry weight organisms/min.) is taken as 1·0 and the other activities expressed relative to it. R_G, R_F, R_S, R_M and R_{GN} denote the levels of activity obtained using glucose, fructose, sucrose, maltose and gluconate respectively as substrates in the enzyme tests. Except where indicated (†) these levels applied at all dilution rates from 0·3 to 1·0 hr^{-1}.

† Maximum values (see text).

Dehydrogenase activities in conditions other than C-limitation

The range of dilution rates from 0·1 to 1·0 hr^{-1} was again used, and when glucose was the carbon source R_G, although lower than in C-limitation, still ran at a high level in K$^+$, NH$_4^+$, Mg^{2+} and SO$_4^{2-}$-limited systems, the constant levels being 76, 73, 72 and 78 % respectively of that in the glucose-limited system, but in the PO$_4^{3-}$-limited conditions it was only 52%. Except in the K$^+$-limited system, however, the range of dilution rates over which R_G was independent of the dilution rate was shorter.

Figures 1 b, c show the results obtained in the K^+ and NH_4^+-limited systems as typical examples, and should be compared with Fig. 1 a. Figures 1 b, c also show that R_F, R_M and R_S were again no longer equal to R_G, but decreased in the order $R_G > R_F > R_S > R_M$. This also occurred in the other nutrient-limited systems, the over-all behaviour once more emphasizing the uniqueness of the equal levels of R_G, R_F, R_S and R_M in the glucose-limited system.

Essentially similar results were obtained when fructose, sucrose or maltose were used in turn as carbon sources. Only K^+ and NH_4^+-limited systems were studied, and again R_G, R_F, R_S and R_M were lower than in the corresponding C-limited systems, even in the special conditions pertaining when the carbon source in the medium and the substrate in the enzyme test were identical. Here the activity of the relevant dehydrogenase increased to almost that of the now lower level of R_G. For example, in sucrose-containing systems, R_G, R_F, R_S and R_M were 83·5, 43·0, 82·2 and 12·3 units respectively in C-limitation, 70·0, 44·2, 55·2 and 2·5 in K^+ limitation, and 52·6, 41·7, 50·4 and 2·5 in NH_4^+-limitation, the order $R_G > R_S > R_F > R_M$ being preserved throughout. Similarly R_F in fructose-limited conditions and R_M in maltose-limited conditions were higher than any other except R_G.

Table 2. *Glucosidase activities in various nutrient-limited systems*

Nutrient controlling growth	Relative activity*	
	α-Glucosidase	β-Glucosidase
(a) Various carbon sources, NH_4^+ as nitrogen source		
Glucose	0·22	0·14
Fructose	0·32	0·14
Sucrose	0·11	0·20
Maltose	1·00	0·08
Cellobiose	0·33	1·00
Melibiose	0·32	0·56
Lactose	0·27	0·15
(b) Glucose as carbon source, NH_4^+ as nitrogen source		
C	0·22	0·14
N	0·08	0·04
K^+	0·06	—
Mg^{2+}	0·15	—
SO_4^{2-}	0·12	—
PO_4^{3-}	0·09	—
(c) Glucose as carbon source, NO_2^- as nitrogen source		
C	0·24	0·72
N	0·13	0·16
(d) Glucose as carbon source, NO_3^- as nitrogen source		
C	0·13	0·01
N	0·30	0·01

* The α-glucosidase specific activity (i.e. 1·96 mμ moles p-nitrophenol liberated/mg. dry weight organisms/min.) of intact organisms grown in the maltose-limited system is taken as 1·0 and the other α-glucosidase activities expressed relative to it. Similarly for β-glucosidases the specific activity obtained in cellobiose-limited conditions (i.e. 36·8 mμ moles p-nitrophenol liberated/mg. dry weight organisms/min.) is taken as 1·0. Dilution rate 0·4 hr^{-1} throughout.

In sharp contrast, the gluconate dehydrogenase specific activity (R_{GN}) of gluconate-grown organisms and the glycerol dehydrogenase specific activity (R_{GL}) of glycerol-grown organisms were approximately twice as high in NH_4^+-limitation compared to

C-limitation (i.e. C-limitation R_{GN} 24·6, R_{GL} 22·0; NH_4^+-limitation R_{GN} 47·7, R_{GL} 46·6). The activities of the other dehydrogenases were also significantly higher except for R_3 in the gluconate system and R_M in the glycerol system which conformed to the more usual pattern. Other nutrient-limited systems were not investigated.

α- and β-glucosidase specific activities

In C-limited conditions in which various sugars were the carbon sources the highest α-glucosidase activity was observed in organisms grown in the maltose (i.e. Glc p α 1 → 4 Glc)-limited system. Melibiose (i.e. Gal p α 1 → 6 Glc) induced little activity and low levels also occurred in glucose, fructose, lactose and sucrose-grown cells, particularly in the last (Table 2*a*). A dilution rate of 0·4 hr^{-1} was used in these experiments since more detailed investigations of glucose and maltose-limited systems had shown that, like dehydrogenase activities in general, α-glucosidase activities were relatively independent of the dilution rate over a wide range. Unlike dehydrogenases, however, the activity increased progressively, rather than decreasing, as the dilution rate was reduced below 0·3 hr^{-1}. Indeed, in the maltose-limited system the levels at rates of 0·2 and 0·1 hr^{-1} respectively were 9·9 and 20·0 % higher than the constant level (compare Fig. 1).

Although low in α-glucosidase, organisms grown in melibiose-limited conditions had a relatively high β-glucosidase activity. This was some 50 to 60 % of the highest level observed, i.e. in the cellobiose (Glc p β 1 → 4 Glc)-limited system, while in the other C-limited systems the activity ranged from about one-third of this optimum level in sucrose-grown organisms to about one-tenth in maltose-grown organisms (Table 2*a*). Table 2*b* shows the lower levels of both glucosidases obtained in NH_4^+-limitation compared to C-limitation when glucose was the carbon source. This also occurred when NO_2^- replaced NH_4^+ as the nitrogen source in the medium but in addition the level of activity of β-glucosidase (but not α-glucosidase) increased considerably in both C and N-limitation (Table 2*c*). Using NO_3^-, however, led to a drop in β-glucosidase activity (Table 2*d*).

DISCUSSION

Clarke *et al.* (1968) found that the specific activity of an inducible amidase in *Pseudomonas aeruginosa* passed through a sharp maximum at a dilution rate of 0·30 to 0·35 hr^{-1} while that of a fully constitutive mutant decreased with increasing dilution rate. On the other hand Wright & Lockhart (1965) reported an increase in the glucose-6-phosphate dehydrogenase activity of *Escherichia coli* K12 with increasing dilution rate. We rarely observed these patterns of behaviour with glucose dehydrogenase. Instead, the specific activity was almost invariably relatively independent of the dilution rate and this, together with the small variations which occurred when many sugars and related substances replaced glucose as the carbon source in the medium (Table 1*a*), could be cited as evidence for a constitutive glucose dehydrogenase system. However the precise differentiation between 'constitutivity' and 'inducibility' can present difficulties (see, for example, Liss, Horwitz & Kaplan, 1962; Moses & Sharp, 1968) and conditions do exist in which the glucose dehydrogenase specific activity is low or even undetectable (Table 1*b*). Moreover even when it remained high over most of the range of dilution rates it decreased at very low rates, and when the

carbon source in the medium was present in excess of requirement, as occurs in all conditions of nutrient-limitation other than C-limitation, the levels of activity were lower throughout.

Apart from gluconate dehydrogenase, which behaved in general like glucose dehydrogenase, the other dehydrogenases, although also relatively independent of the dilution rate, were much more affected by the nature of the rate-limiting nutrient and appeared to increase in activity just when required. This is the behaviour of inducible dehydrogenase systems and is in agreement with the conclusions drawn by others from batch culture experiments using a variety of organisms (see Lin, 1961; Hayashi, Hayash & Unemoto, 1966; Henning, Dietrich & Deppe, 1968). However, Liss et al. (1962) and Tanaka, Lerner & Lin (1967) concluded that *Klebsiella aerogenes* (ATCC 8724) dissimulated mannitol via a constitutive mannitol-1-phosphate dehydrogenase. Mannitol-grown organisms had an increased level of this enzyme but in contrast to our strain showed no mannitol dehydrogenase activity. Schaefler & Schenkein (1968) and Fox & Wilson (1968) envisage that phosphorylation also precedes the hydrolysis of β-glucosides in Enterobacteriaceae, in general, since the liberation of *p*-nitrophenol from phosphorylated *p*-nitrophenol β-D-glucosides was more rapid than from the non-phosphorylated substrates. We have used the latter throughout our assays for α- and β-glucosidases and these enzymes also increased in activity just when required, although the induction of β-glucosidase by melibiose (Gal p α 1 → 6 Glc) is exceptional in this respect. Melibiose also induces β-galactosidase (Monod, Cohen-Bazire & Cohn, 1951).

Variations in the metabolism of glucose by different strains of *Klebsiella aerogenes* have also been reported. Forget (1968) estimated, from tests carried out after 20 to 24 hr of growth in aerobic batch culture, that the Embden-Meyerhof and the hexose-monophosphate shunt pathways participated to the extent of 60 and 40 % respectively in strain L 111-1. In contrast, in *K. aerogenes* (PRL-R3) Dalby & Blackwood (1955) obtained evidence for the operation of a non-phosphorylated Entner-Doudoroff type of metabolic pathway, in which glucose is dehydrogenated to gluconate by glucose dehydrogenase. In our experiments the correlation between a high level of glucose dehydrogenase activity and an appreciable gluconate dehydrogenase activity suggests that a similar pathway exists in *K. aerogenes* (NCIB 418) and an approximate calculation based on the transfer of electrons to TTC shows that under optimum conditions 40 % of the glucose in the medium could be degraded by this route, the remainder presumably being metabolized by more usual routes, although no direct evidence of those involved is available at present. Similar considerations could also apply in the other chemostat systems in which the activity of the relevant dehydrogenase is high although variations in some of the steps in the reaction sequences are inevitable. In particular, disaccharides could either be dehydrogenated directly (Gunsalus, Horecker & Wood, 1955) or else hydrolysed before further degradation, and in some circumstances this hydrolysis might conceivably occur during the dehydrogenase tests themselves. For example, organisms from the maltose-limited system have a high α-glucosidase activity and since in the enzyme tests bacteria and substrate were incubated together before adding the TTC, it is quite possible that part at least of the estimated maltose dehydrogenase activity is due to the action of glucose dehydrogenase. However, in other systems such as the NH_4^+ and K^+-limited maltose systems, the α-glucosidase activity of the organisms is low and the dehydrogenation of the substrate itself would be measured.

Definite proof of the presence of a specific maltose dehydrogenase system, and of the others also, requires the isolation of the enzymes themselves and for this a gentler method of cell rupture is desirable since the use of any of a variety of standard techniques led to marked inactivation. This also precluded any assessment of permeation effects by a comparison of the activities of intact and disrupted organisms. Nevertheless, incubation of the intact organisms with substrate prior to adding the TTC resulted in maximum activity and on its addition the response was linear although when the activities were low this was preceded by a lag. Grant & Hinshelwood (1964) concluded that permeation of the substrate was not the rate-limiting step with 'fully mobilized' dehydrogenases in our strain of *Klebsiella aerogenes* grown in batch culture, although it might be with 'non-mobilized' dehydrogenases. As in our experiments they observed a decline in activity of the former on ultrasonic treatment of the bacteria, whereas the low levels of activity of the 'non-mobilized' enzymes increased some 50 % before decreasing. We have not investigated this latter aspect since, being obliged to use intact organisms when the activities were high, a standard technique was used throughout. Effects of this magnitude, however, do not in themselves account for the low levels of activity. The precise conditions of growth are of greater importance.

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Metabolism of Macromolecules in Bacteria Treated with Virginiamycin

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SUMMARY

The two components of virginiamycin, M and S, separately exerted a reversible bacteriostatic activity on *Bacillus subtilis*. Their combination increased by a hundredfold the inhibitory activity of each factor and induced a loss of viability of bacteria. Such an irreversible step was preceded by a reversible phase, which was characterized by a long lag in colony formation.

Very short incubation with single virginiamycin components and their combination suddenly and completely blocked protein synthesis, whereas the rate of incorporation of labelled bases and nucleosides into polynucleotides was not altered appreciably unless protein formation was halted completely.

Nevertheless, some alterations of ribosomal RNA metabolism occurred very early after treatment with virginiamycin. The synthesis of 23S rRNA was specifically inhibited. Moreover, the degree of methylation of the rRNA which was made in the presence of the drug was lower than that of the controls. Also, the rRNA labelled in virginiamycin-treated cells was metabolically unstable. This indicates that formation and stability of rRNA, as well as the balance among rRNA species, depend on virginiamycin-sensitive protein synthesis.

Metabolism of pulse-labelled RNA was also altered in the presence of virginiamycin: its half-life was prolonged about sixfold by single components and eightfold by their combination. This was due to an increased turnover of rRNA and to prevention of messenger RNA decay.

It is concluded that peptide chain formation is the primary target of virginiamycins M and S (hence their synergistic antibiotic activity), that translation—not transcription—is prevented by these inhibitors, and that the alterations of nucleic acid metabolism are due to the halt of protein synthesis.

INTRODUCTION

Virginiamycin, an antibiotic which is produced by a mutant of *Streptomyces virginiae*, inhibits the growth of Gram-positive organisms *in vitro* and *in vivo* (Vanderhaeghe, van Dijck, Parmentier & De Somer, 1957). Its biological and chemical properties resemble those reported for other complex inhibitors which were described as Mikamycin, Ostreogrycin or E129 complex, PA114 factor, Pristinamycin or Pyostacin, Streptogramin and Vernamycin. The common feature of these antibiotics is that they contain two factors which, though exerting different degrees of inhibition on various micro-organisms, do possess a synergistic action in a given bacterium (Vazquez, 1966*a*).

Factor 'M' of virginiamycin, which is particularly active on micrococci, is a

macrocyclic lactone containing an oxazole ring (Fig. 1): it is probably identical to Ostreogrycin A (Vanderhaeghe et al. 1957; Delpierre et al. 1966). Factor 'S' of virginiamycin has a more powerful activity on bacilli and consists of a cyclopeptidic lactone ring (Fig. 1) (Vanderhaeghe & Parmentier, 1960) which resembles that of the B factor of Ostreogrycin complex (Eastwood, Snell & Todd, 1960) and of the I_A component of Pristinamycin (Jolles, Terlain & Thomas, 1965).
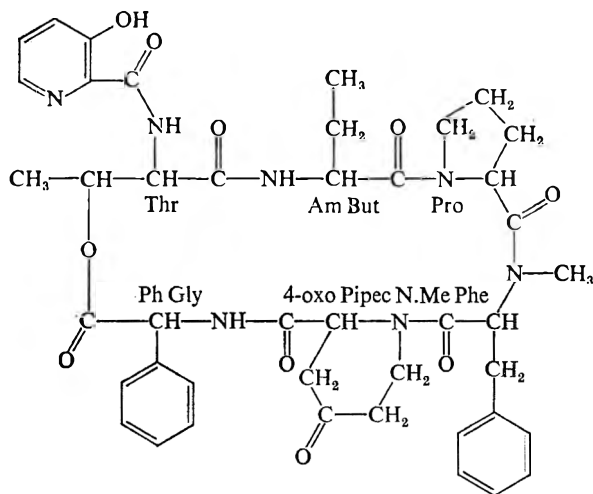
Virginiamycin S
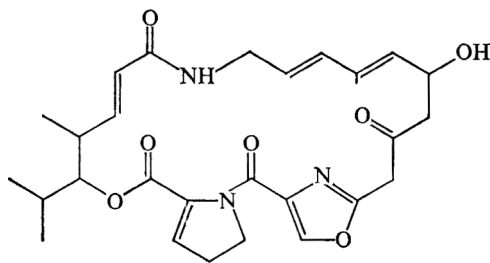
Virginiamycin M

Fig. 1. Chemical structure of virginiamycin components.

The mechanism of the inhibitory activity of these complex antibiotics, which has been described respectively as bacteriostatic (Yamaguchi & Tanaka, 1964) and bactericidal (Chabbert & Acar, 1964; Videau, 1965; Murat & Pellerat, 1965), is still unclear. In several respects they resemble chloramphenicol, particularly in their ability to inhibit in some micro-organisms the incorporation of amino acids into polypeptides without altering the incorporation rate of radiophosphorus into polynucleotides (Yamaguchi & Tanaka, 1964).

However, a deeper investigation has revealed differences in the action of the two

components of streptogramin complex and chloramphenicol. Thus, for example streptogramin A (which corresponds to virginiamycin M) reduces the fixation of chloramphenicol to bacteria and ribosomes, and likewise chloramphenicol represses protein—not nucleic acid—formation in intact cells. Nevertheless, when tested *in vitro* on amino acid incorporation directed by synthetic polynucleotides, streptogramin A and chloramphenicol show different patterns of inhibition. On the other hand, streptogramin B (which resembles virginiamycin S) reduces the uptake of chloramphenicol by intact bacteria but does not compete with the chloramphenicol binding to ribosomes. Moreover, streptogramin B shows *in vitro* the same pattern of inhibition as chloramphenicol on homopolymer-directed amino acid incorporation, but in sensitive cells inhibits the accumulation of both nucleic acids and proteins (Vazquez, 1966*a*, *b*, *c*, *d*).

To reconcile such conflicting results one must postulate that streptogramin and chloramphenicol have more complex activities and interfere with several steps of the synthetic pathways of nucleic acids and proteins. The aim of this study was to elucidate the mechanism of action of virginiamycin complex in uninfected and virus-infected cells. The present and the accompanying paper deal respectively with the action of virginiamycin in *Bacillus subtilis* and in bacteria infected with the DNA-bacteriophage 2C. Purification of virginiamycins M and S and preparation of their labelled derivatives have made this investigation feasible.

METHODS

Abbreviations used: rRNA, mRNA and tRNA = ribosome, messenger and transfer RNA; HMW-RNA and LMW-RNA = high- and low-molecular weight RNA (chromatography fractions); Leu = leucine; Trp = tryptophan; Glc = glucose; U = uridine; T = thymidine; ECD = enzymic digest of casein; BSA = bovine serum albumin; TCA = trichloroacetic acid; EDTA = ethylene diaminetetraacetic acid; SDS = sodium dodecyl sulphate; DOC = sodium deoxycholate; MAK = kieselguhr coated with methylated albumin; Klett Units (K.U.) = $A_{660 m\mu} \times 10^3/2$; L and OS = media for *Bacillus subtilis*.

Micro-organisms. Three strains derived from *Bacillus subtilis* 168 were used in different experiments: (1) 168/6, wild type; (2) 168/2 leu⁻ and trp⁻; and (3) A26 U⁻ and trp⁻. *Escherichia coli* strain B furnished the RNA to be used as heterospecific marker for ultraviolet absorption.

Growth media. Stock cultures were grown in L medium containing 10 g. tryptone, 10 g. yeast extract, 5 g. NaCl, and 10^{-5}M-MnCl$_2$ per litre. Experiments employing either [³H]uridine (strains 168/2 and A26), [¹⁴C]amino acids (strains 168/2 and A26), or [¹⁴C]glucose (168/6) as tracers were carried out in a high-phosphate OS medium (Spizizen, 1958) supplemented with different amounts of Glc, ECD, Leu, Trp and U. Three different media (Young & Spizizen, 1963; Schaechter, 1963; Yudkin & Davis, 1965) were used to label cells with [³²P]phosphate: the level of [³¹P]phosphate was lowered to 10^{-3} M and replaced with 0·05 M-tris-HCl buffer (pH 7·4.)

Evaluation of bacterial numbers and viability. Bacterial concentration was evaluated by turbidimetric determination at 660 mμ in a Klett Summerson photoelectric colorimeter. Viability was determined by appropriate dilution in DSP (8·7 g. NaCl, 3·5 g, K$_2$HPO$_4$, 0·5 g. peptone, 10^{-6}M-MnCl$_2$ (pH 7·5) adjusted with HCl) and plating on L

agar; colonies were counted after different times of incubation at 37°. As a check on turbidimetric units and colony-forming counts, microscope counts (after fixation and staining with 5 % I in 10 % KI) and DNA content were previously established.

Isolation and disruption of bacteria. Three techniques were followed to stop metabolism while harvesting bacteria from growth medium: (1) NaN$_3$ (10^{-2} M) and rapid cooling followed by centrifugation; (2) precipitation with ice-cold TCA (0·5 %, w/v, f.c.) and filtration on micropore filters; (3) precipitation with 95 % ethanol at −18°.

Colorimetric determinations and radioactivity measurements were carried out directly on the acid and alkali extracts. Isolation and separation of macromolecules were carried out on homogenates obtained by mechanical disruption in a French-Aminco cell or by enzymic lysis with crystalline egg lysozyme in 0·15 M-NaCl, 0·1 M-tris-HCl (pH 7·4)+0·02 M-NaN$_3$. *Escherichia coli* RNA was extracted directly from intact organisms, with a hot phenol+detergent procedure (Cocito & De Somer, 1961; Cocito, 1963).

RNA extraction. Homogenates of *Bacillus subtilis* were treated with SDS (0·25 %, w/v) and freshly redistilled phenol, the mixtures were centrifuged and the aqueous layers were re-extracted with phenol and ether. Suitable amounts of *Escherichia coli* RNA and NaCl (0·5 M) were added, and RNA was precipitated from 95 % ethanol at −18°.

Ultracentrifugal fractionation of RNA. Solutions of RNA were layered on the top of 15 to 30 % (w/v) gradients of sucrose in 0·10 M-NaCl, 0·2 M-acetic acid/Na acetate buffer (pH 5·2). Depending on the temperature of the centrifuge (10 to 23°) 0·1 to 2·5 mg. SDS/ml. were added. Fractionation was carried out either in a Spinco (rotor SW 25·1, 23,000 rev./min.) or in an MSE ultracentrifuge (swing-out rotor 30, 30,000 rev./min.).

Radioactivity measurements. To ice-cold solutions of labelled nucleic acids or proteins 50 µg. bovine serum albumin and TCA (0·3 M) were added. After 30 min. of incubation in melting ice, the samples were filtered through 0·45 µ micropore membranes pre-soaked in 0·3 M-TCA for 18 hr at 4°. Precipitates were washed on the filters with ice-cold 0·3 M-TCA and air dried. Samples for ^{3}H, ^{14}C and ^{32}P were counted in a refrigerated liquid scintillation counter (Nuclear Chicago). Toluene containing 4 g. 2,5-diphenyloxazole (PPO) and 0·1 g. 1,4-bis-(5-phenyloxazolyl-2-)benzene (POPOP) per litre was used as scintillation fluid for solid samples, and Bray (1960) solution for liquid samples.

Colorimetric determinations. Phosphorus determinations were carried out with a modification of Fiske & SubbaRow procedure (Lindberg & Ernster, 1956). The colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used for proteins. DNA was determined with a modification of the Dische-Burton diphenylamine reaction (Giles & Myers, 1965). The colorimetric analysis of RNA was carried out with the orcinol method of Mejbaum modified by Ceriotti (1955).

Chemical fractionation of whole bacteria and cellular homogenates. Bacterial suspensions and homogenates were treated with TCA (0·5 M, 4°) and centrifuged. The pellets were dissolved in 0·1 M-NaOH. One sample of the solution was used for colorimetric estimation of proteins, and another for nucleic acid determination. The latter was performed by precipitation with HCl and 0·3 M-TCA at 4°, centrifugation, extraction of the sediment with HClO$_4$ for 15 min. at 90° and incubation in ice for 30 min. after addition of 2 mg. BSA. The precipitate of proteins was removed by

centrifugation and DNA and RNA were determined colorimetrically in the supernatant.

Purification of virginiamycins. Separation and purification of M and S factors from crude virginiamycin preparations was achieved on columns of silica gel (Vanderhaeghe *et al.* 1957; Vanderhaeghe & Parmentier, 1960). Crystalline preparations of the two factors (Gosselinckx, 1963) were employed through the entire work. The corresponding aqueous solutions were freshly prepared before each experiment.

Chemicals. Most of the radioactive products were obtained from the Belgian and French Departments of Radioisotopes of C.E.N. and the Radiochemical Centre, Amersham, England. Nucleic acid components were obtained from Calbiochem and Sigma, nucleases from Worthington, bovine serum albumin (fraction V) from Armour and Na laurylsulphate from T. Schuchard. The antibiotics actinomycin D and virginiamycin were gifts from Merck Sharp & Dohme (U.S.A.) and R.I.T. (Belgium(respectively: they are trade marked compounds of these two companies.

RESULTS

Action of virginiamycin on growth and viability of Bacillus subtilis. Growth curves of *Bacillus subtilis* 168/2 with increasing amounts of a single virginiamycin component are reported in Fig. 2. Factor S was more active than factor M, on a weight basis. Mixing the two virginiamycin components potentiated their antibiotic activity about 200-fold. In fact, a level of 0·5 μg./ml. of factors M and S induced an inhibitory effect comparable to that of 100 μg. of a single component.

The numbers of colony-forming units after incubation for different periods with virginiamycin components or their combination is reported in Table 1. Incubation of bacteria with high levels of single factors for less than 2 hr did not reduce their colony-forming capacity after plating on L agar (the viability decreased slowly as the cultures were maintained at 37° for longer periods). Mixing the two virginiamycins produced a double effect. For exposures shorter than 2 hr, the bacteria retained their viability, but colonies appeared on agar plates after an abnormal lag. Exposure times longer than this caused the colony-forming capacity to decrease rapidly. Hence, single factors had a bacteriostatic activity while together they were bactericidal, with an initial period in which the colony-forming capacity was altered.

Kinetics of amino acid incorporation into polypeptides. Incorporation of [^{14}C]amino acids into acid-insoluble polypeptides was followed in organisms which were incubated with factors M, S or both. Both factors reduced the incorporation rate with little lag (Fig. 3) and the pattern of inhibition remained unchanged over a three-generation period. The rapidity of virginiamycin action was emphasized by slowing down the growth rate using auxotrophs grown in low amino acid medium. Under these conditions the inhibition by 2·5 μg. of M or S factors was more than 80% within 5 min. and amino acid incorporation was virtually halted within 1/25th of the generation time.

Rate of incorporation of labelled precursors into nucleic acids. Is polypeptide synthesis the direct target of the drug or the result of an inhibition of nucleic acid synthesis? The problem was investigated by following the rate of incorporation of labelled precursors into polynucleotides, in the presence of virginiamycins.

In the prototroph strain 168/6, virginiamycin M and a mixture of M and S

factors increased the incorporation rate of [³H]thymidine into polydeoxyribonucleotides, and prolonged it beyond the limit of the control (Fig. 4). The same precursor was incorporated at a reduced rate upon incubation of the auxotroph mutant A26 with virginiamycins; this inhibition was negligible for the first 25 min. and increased as the time of contact with the drug was prolonged. The rate of incorporation of labelled uracil and uridine into polyribonucleotides was either unimpaired or increased during the first 15 min. of incubation with virginiamycin and decreased rapidly afterwards (Fig. 5).

Table 1. *Colony-forming units and time of colony formation after growth in the presence of virginiamycin components*

Strain and medium as in the legend for Fig. 2. Virginiamycin M (10 and 100 μg./ml.), S (same) and M+S (0·5 and 5 μg. each component/ml.): added at time 0 to the cultures. Viability test: samples taken at different intervals after addition of the antibiotic, immediately diluted and plated on nutrient agar. Visible colonies counted after 14, 24, and 48 hr at 37°.

Time of contact with Virginiamycin (min.)	Time of incubation of agar plates (hr)	= control	10 μg. M_1	100 μg. M_1	10 μg. S	100 μg. S	0·5 μg. M_1 +0·5 μg. S	5 μg. M_1 +5 μg. S
0	14	$1·64 \times 10^7$						
	24	$1·64 \times 10^7$						
10	14		$2·56 \times 10^{7*}$	$2·48 \times 10^7$	$4·88 \times 10^7$	$3·40 \times 10^7$	—	—
	24		$2·56 \times 10^{7*}$	$2·48 \times 10^7$	$4·88 \times 10^7$	$3·40 \times 10^7$	$3·26 \times 10^7$	$3·08 \times 10^{7*}$
25	14	$6·8 \times 10^7$						
	24	$6·8 \times 10^7$						
30	14		$3·90 \times 10^7$	$4·40 \times 10^7$	$4·10 \times 10^7$	$5·70 \times 10^7$	—	—
	24						$3·70 \times 10^7$	$3·32 \times 10^7$
60	14		$5·0 \times 10^7$	$5·60 \times 10^7$	$5·66 \times 10^7$	$5·28 \times 10^7$	—	—
	24		$5·0 \times 10^7$	$5·60 \times 10^7$	$5·66 \times 10^7$	$5·28 \times 10^7$	$2·60 \times 10^7$	$2·36 \times 10^7$
65	14	$14·0 \times 10^7$						
	24	$14·0 \times 10^7$						
90	14		$7·02 \times 10^7$	$5·80 \times 10^7$	$8·60 \times 10^7$	$8·00 \times 10^7$	—	—
	24						$2·20 \times 10^7$	$3·04 \times 10^7$
120	14		$9·0 \times 10^7$	$5·80 \times 10^7$	$8·04 \times 10^7$	$7·70 \times 10^7$	—	—
	24		$9·0 \times 10^7$	$5·80 \times 10^7$	$8·04 \times 10^7$	$7·70 \times 10^7$	$1·84 \times 10^7$	$2·46 \times 10^7$
145	14		$11·0 \times 10^7$	$4·9 \times 10^7$	$6·1 \times 10^7$	$6·0 \times 10^7$	—	—
	24						$1·82 \times 10^7$	$1·60 \times 10^7$
170	14		$7·0 \times 10^7$	$3·5 \times 10^7$	$4·4 \times 10^7$	$3·2 \times 10^7$	—	—
	24		$7·0 \times 10^7$	$3·5 \times 10^7$	$4·4 \times 10^7$	$3·2 \times 10^7$	$1·56 \times 10^7$	$1·50 \times 10^7$

* Number of colonies of size comparable to that of the control, after incubation of nutrient agar plates for either 14 or 24 hr at 37°.

Synthesis of metabolically stable RNA. Though the halt of polypeptide synthesis cannot be accounted for by a block of polynucleotide chain formation, the effect of virginiamycin on proteins might still be caused by an inhibition of the synthesis o a single nucleic acid species. To test this possibility, RNA was labelled for 15 min. with [³H]uracil (or uridine), extracted and fractionated by centrifugation in density gradients. In exponentially growing bacteria most of the label of a 15 min. pulse was incorporated into metabolically stable rRNA and tRNA (Fig. 6A).

The initial effect of virginiamycins showed a main radioactivity peak overlapping that of the 16S component, very little with 23S rRNA and more label in the 10S and 4S regions (Fig. 6B). After prolonged incubation with virginiamycins the 16S rRNA peak was still higher and broader than that of 23S rRNA, the proportion of RNA radioactivity in the 4S peak was higher than in the controls, and material of high specific activity which sedimented ahead of the rRNAs accumulated (Fig. 6C).
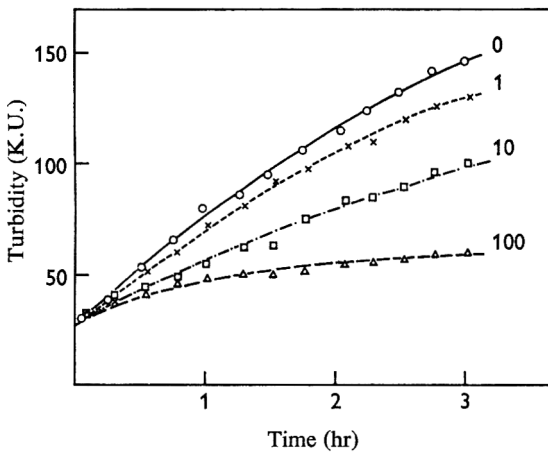

Fig. 2

Fig. 3

Fig. 2. Kinetics of growth in the presence of virginiamycin. Strain 168/2. Medium: OS, supplemented with 5 mg. Glc, 4 μg. Trp, 8 μg. Leu and 800 μg. ECD/ml. Virginiamycin S (1, 10 and 100 μg./ml.) added at time 0. Growth of the agitated culture at 37° followed turbidimetrically (K.U.).

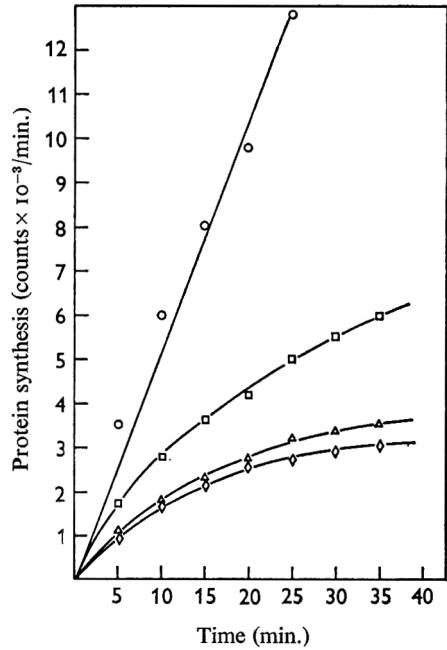Fig. 3. Kinetics of incorporation of amino acids into proteins. Strain A26. Medium: OS, supplemented with 5 mg. Glc, 4 μg. Trp, 4 μg. Leu, 200 μg. ECD, and 100 μg. U/ml. Virginiamycin: none (control) O—O; 50μg. factor M —△—; 50 μg. factor S —□—; 2·5 μg./ml. of both components —◇—, added at −1 min. to the cultures. Labelling: [^{14}C]algal protein hydrolysate, specific activity 520·8 μc/mg., added at time 0 (0·417 μc/ml., f.c.). TCA-insoluble radioactivity collected on micropore filters and counted.

Integrated values of radioactivity of RNA fractions (which were pooled according to the scheme in Fig. 6A) are summarized in Table 2. During the first 15 min. of contact with virginiamycins M and M+S, incorporation of uracil into every type of RNA was increased. As the period of growth in the presence of virginiamycins was prolonged, however, the incorporation rate decreased progressively. In addition, the ratio 4S RNA/16S+23S RNA increased in proportion to the length of exposure to the drug, thus indicating a possible turnover of the high molecular weight fraction. Similar results were obtained by fractionation on MAK columns of RNA labelled with [^{32}P]orthophosphate.

Data reported in this section show that over-all synthesis of rRNA and tRNA was inhibited only after prolonged incubation with virginiamycin and exclude the possibility that the halt of protein synthesis might be due to a block of the transcription mechanism. Yet, virginiamycin induced a rapid alteration of RNA metabolism, as indicated by: (a) an early interference with the formation of 23S rRNA, (b) the accumulation of RNA with sedimentation coefficient higher than 23S, and (c) a probable turnover of rRNA. These alterations did not occur in rRNAs which were labelled before transfer to unlabelled medium containing virginiamycin (Fig. 7).

Time (min.)   Time (min.)
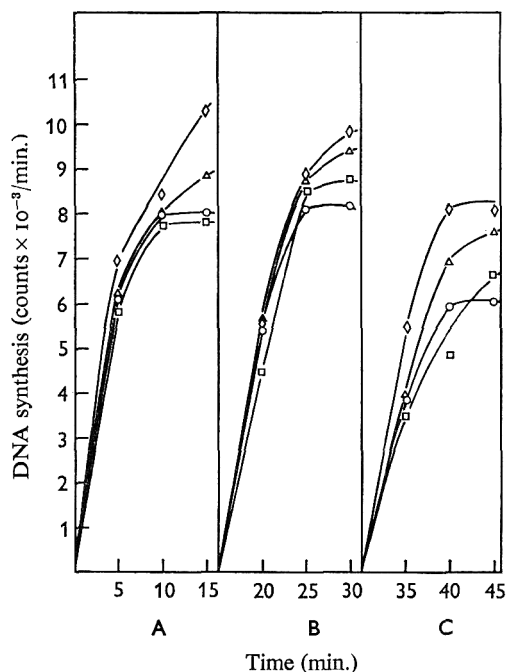
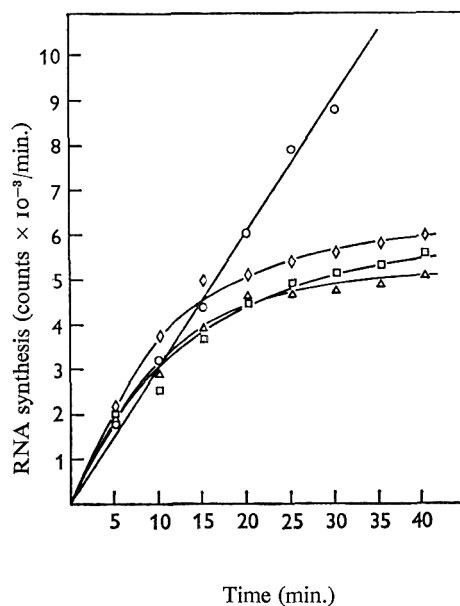Fig. 4   Fig. 5

Fig. 4. Rate of labelling of DNA in a virginiamycin-treated prototroph. Strain 168/6. Medium: OS, supplemented with 5 mg. Glc and 800 μg. ECD/ml. Virginiamycin: none (control) O—O; 50 μg. factor M —△—; 50 μg. factor S —□—; 2·5 μg./ml. of both components —◇—. Labelling: [^{3}H]thymidine, specific activity 17 c/mmole, added at o (A), 15 (B) and 30 (C) min. to the cultures (o·113 μc/ml., f.c.). Sampling: every 5 min. for 15 min. TCA-insoluble radioactivity collected on micropore filters in the presence of 100 μg. unlabelled thymidine and BSA/ml. and counted.

Fig. 5. Kinetics of incorporation of uracil into RNA. Strain and medium as in the legend for Fig. 3, but 800 μg. ECD/ml. Virginiamycin: none (control) O—O; 50 μg. factor M —△—; 50 μg. factor S —□—; 2·5 μg./ml. of both components —◇— added at −1 min. to the cultures. Labelling: [6-^{3}H]uracil, specific activity 12 c/mmole, added at time o (o·417 μc/ml., f.c.). TCA-insoluble radioactivity, precipitated in the presence of 100 μg. of unlabelled uracil and 100 μg. of BSA/ml. collected on micropore filters, and counted.

Turnover of pulse-labelled RNA in virginiamycin-treated cells. Bacteria were treated with virginiamycins, labelled with pulses of [^{3}H]uridine of 45 sec. and chased with an excess of unlabelled uridine and actinomycin (to prevent re-incorporation). At different times in the labelling and chasing periods, samples of the cultures were withdrawn, and the radioactivity incorporated into RNA was precipitated and measured.

Factor M apparently increased the level and inhibited the decay of pulse-labelled RNA: mean values of 90 and 420 sec. were calculated for the half-lives of pulse-labelled RNAs from control and M-treated bacteria respectively (Fig. 8). The half-life of RNA pulse-labelled in the presence of factor S was about 360 sec., whereas an eight-fold increase was caused by a combination of virginiamycin M and S.

Table 2. *Synthesis of rRNA and tRNA in the presence of virginiamycin*

Strain and medium as in the legend for Fig. 4. Virginiamycin: none (control); 50 µg. factor M; 50 µg. factor S; and 2·5 µg. both factors/ml., added 3 min. before the isotopes. Labelling: [6-^{3}H]uracil (specific activity 12 c/mmole, 1·04 µc/ml., f.c.) added at 15 min. interval to three sets of samples. Bacteria labelled with 15 min. pulses were disrupted by compression, treated with DNAse and extracted with phenol. RNA was fractionated in a sucrose density gradient. Absorbance (A$_{265\,m\mu}$) was continuously monitored, and TCA-insoluble radioactivity of fractions, pooled according to the scheme of Fig. 6A, was collected on micropore filters and counted.

Virginiamycin*		Labelling†	Turbidity‡	Radioactivity incorporated into RNA (acid-insoluble counts/min. × 10^{-3} [^{3}H]uracil × ml.$^{-1}$)							Ratios % age var. total RNA	rRNA/tRNA
Factor	(µg./ml.)	(min.)	(K.U.)	>23S (A)	23S (B)	16S (C)	10S (D)	4S (E)	<4S (F)	Total (G)	(H)	(I)
—	—	2–17	51	2·92 (3·0)	25·17 (26·0)	19·82 (20·5)	13·05 (13·5)	25·41 (26·3)	10·30 (10·6)	96·67 (100)	—	1·77
M	50	—	35	10·65 (6·7)	33·79 (21·1)	31·28 (19·6)	25·76 (16·1)	45·49 (28·4)	12·59 (7·9)	159·56 (100)	+65·1	1·43
S	50	—	34	4·65 (6·3)	12·97 (17·4)	15·93 (21·5)	10·26 (13·8)	26·18 (35·3)	4·25 (5·7)	74·24 (100)	−23·2	1·10
MS	2·5	—	36	14·66 (6·3)	45·32 (19·3)	41·12 (17·6)	28·76 (12·3)	87·87 (37·5)	16·65 (7·1)	234·38 (100)	+142·5	0·98
—	—	14–29	64	5·64 (3·5)	47·81 (29·9)	30·81 (19·3)	27·42 (17·1)	39·17 (24·5)	9·83 (6·2)	160·68 (100)	—	2·01
M	50	—	49	4·40 (4·9)	13·67 (15·1)	23·13 (26·0)	17·66 (19·9)	25·31 (28·5)	4·73 (5·3)	88·90 (100)	−44·7	1·45
S	50	—	48	4·12 (5·0)	14·66 (17·5)	15·95 (19·2)	14·32 (17·3)	29·60 (35·7)	4·38 (5·3)	83·03 (100)	−48·3	1·03
MS	2·5	—	51	8·83 (5·1)	31·30 (17·7)	47·36 (26·8)	25·58 (14·5)	57·40 (32·5)	6·29 (3·6)	176·76 (100)	+10·1	1·37
—	—	22–37	77	3·15 (1·5)	71·32 (34·1)	48·08 (23·1)	24·28 (11·6)	46·30 (22·2)	15·58 (7·5)	208·71 (100)	—	2·58
M	50	—	57	10·57 (11·9)	22·55 (25·5)	21·29 (24·1)	7·41 (8·4)	18·44 (20·9)	8·06 (9·1)	88·32 (100)	−57·6	2·38
S	50	—	58	6·91 (10·8)	15·83 (24·7)	12·17 (19·0)	7·30 (11·4)	17·95 (28·0)	3·93 (6·1)	64·09 (100)	−69·3	1·56
MS	2·5	—	61	10·85 (23·8)	7·16 (15·7)	4·53 (10·0)	7·67 (16·9)	13·04 (28·6)	2·22 (4·9)	45·47 (100)	−78·2	0·90

* Experimental conditions as in the legend for Fig. 8A. † Length of labelling period after addition of virginiamycin. ‡ Turbidity of the harvested culture. (H) Percentage variation of total acid-insoluble polynucleotides (G) with respect to the control. (I) (B+C)/E. In parentheses = percentage distribution of total RNA radioactivity (G) among RNA peaks.

Whilst there were differences in the turnover rates of different species of RNA, the ultracentrifugal patterns of RNA extracted from pulse-labelled virginiamycin-treated bacteria did not differ basically from those obtained in the absence of the anti-
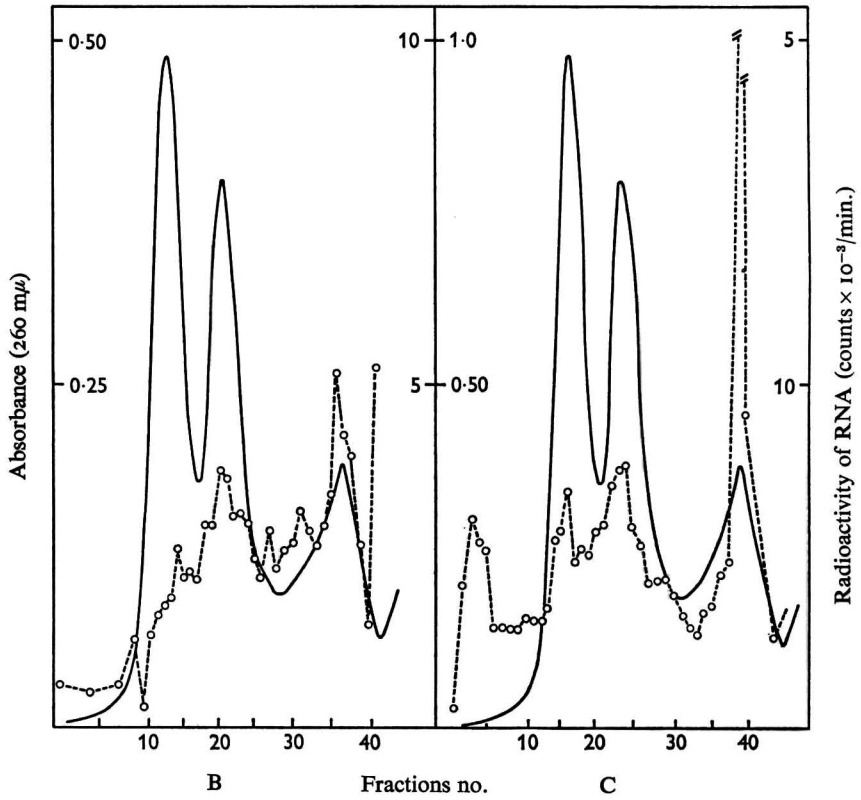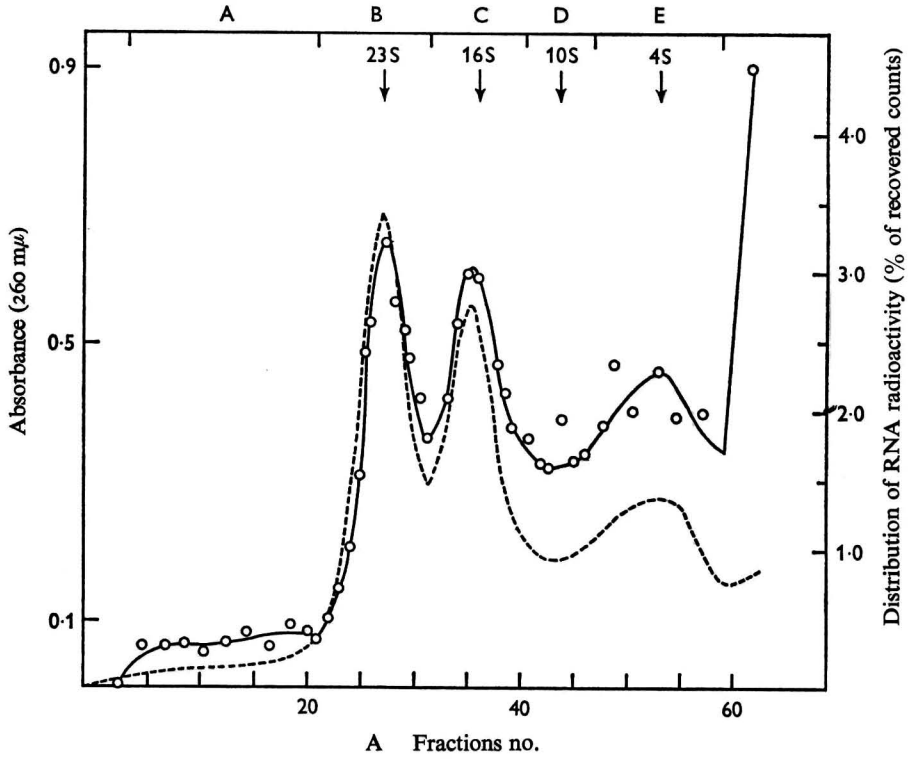
Fig. 6. For legend see foot of facing page.
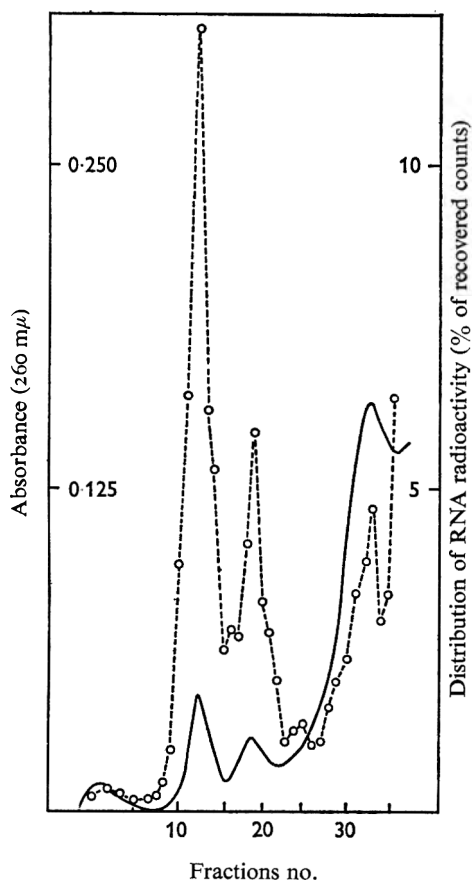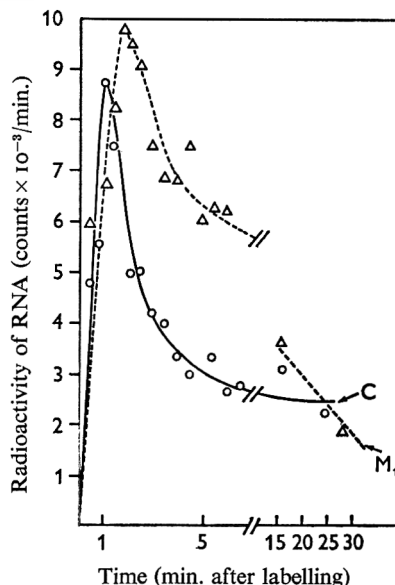
Fig. 7   Fig. 8

Fig. 7. Metabolic stability of RNA labelled before incubation with virginiamycins. Strain and medium as in the legend for Fig. 5. Labelling: growth for 2 generations in the presence of [³H]uridine (specific activity 37·3 mc/mg., 5 μc/ml., f.c.). Harvested bacteria were grown for two generations in unlabelled medium containing 200 μg. of uridine/ml. To a sample of the culture 50 μg. factor M/ml. were added, and growth was allowed to continue at 37°. Samples of the cultures were withdrawn at intervals for nucleic acid analysis, and a sample collected after 30 min. of contact with the antibiotic is shown. Other details as for Fig. 6.

Fig. 8. Synthesis and turnover of pulse-labelled RNA. Strain and medium as in the legend for Fig. 2. Control O—O; virginiamycin M 100 μg./ml. —△—, added 10 min. before the isotopes. Labelling (time 0): [6-³H]uracil (specific activity 12 c/mmole, 2·86 μc/ml., f.c.). Chasing (at 45 sec.): 100 μg. of unlabelled uracil and 10 μg. actinomycin D/ml. TCA-insoluble radioactivity precipitated in the presence of 200 μg. unlabelled uracil and 100 μg. BSA/ml., collected on micropore filters and counted.

Fig. 6. Fractionation in sucrose density gradients of RNA labelled in the presence of virginiamycin. Strain and medium as in the legend for Fig. 4. A: no virginiamycin. B and C: 50 μg. virginiamycin M. Labelling: [6-³H]uracil (specific activity 12 c/mmole) added to the cultures (1·04 μc/ml., f.c.) either 3 min. (A and B) or 30 min. (C) after virginiamycin. Bacteria were harvested by centrifugation 15 min. after addition of isotopes, disrupted by compression, treated with DNAase and extracted with phenol and ether. RNA was fractionated by centrifugation in sucrose gradient, absorbance at 260 mμ was continuously monitored, and TCA-insoluble radioactivity of the fractions was collected on micropore filters and counted.

biotic. In both cases most of the label of a 1 min. pulse was found in rRNA precursors, and little of it in tRNA, as shown by the overlapping of the curve of radioactivity and the 23S and 16S absorbance peaks. The main difference was that the amount of labelled precursors incorporated into the 23S rRNA peak was proportionally lower in the presence than in the absence of staphylomycin.

Virginiamycin thus alters the turnover—not the formation—of pulse-labelled RNA, although some early alterations of the synthesis of 23S precursor RNA could be observed in bacteria incubated with the antibiotic.
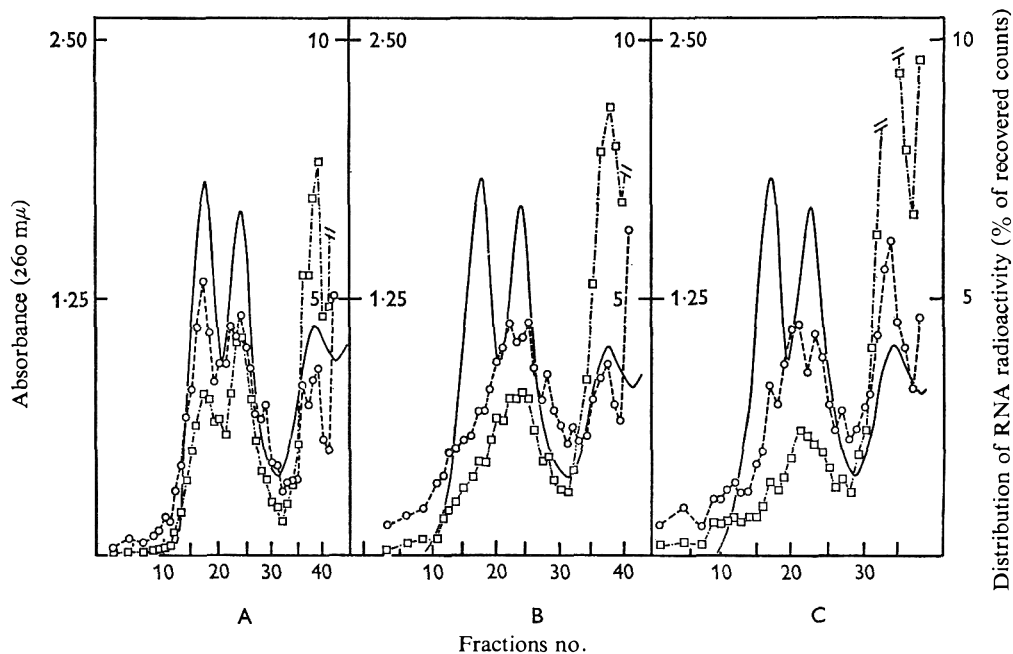

Fig. 9. Ultracentrifugal fraction of methylated and total RNA, labelled and chased in the presence of virginiamycins. Strain A26. Medium: OS, supplemented with 5 mg. Glc, 4 μg. Trp, 8 μg. Leu, 40 μg. U and 200 μg. ECD/ml. A: control. B and C: 50 μg. virginiamycin M/ml. (added at time o). Eight min. pulses of [6-^{3}H]uracil (specific activity 10 c/mmole, 3·130 μc/ml., f.c.) and [^{14}C]methyl-L-methionine (specific activity 24·6 mc/mmole, 0·156 μc/ml., f.c.) given 3 min. later. At the end of labelling period one sample of the culture was cooled with dry ice and treated with NaN$_3$ (B); another was chased for 10 min. with 200 μg. of uracil of methionine and 1 mg. of ECD/ml. (C). Harvested bacteria were lysed enzymically, digested with DNAse, and extracted with SDS and phenol at 4°. RNA was precipitated and then fractionated as in the legend for Fig. 6 and TCA-insoluble ^{14}C (—□—) and ^{3}H (—○—) radioactivities of the fractions were counted and plotted together with the absorption tracing (—).

Metabolism of methylated RNA in the presence of virginiamycins. Data presented in the previous sections indicate that bulk RNA made in the presence of virginiamycins might be unstable and undergo turnover. More direct evidence for turnover was gathered by following the fate of methylated RNA. Since rRNA is methylated after formation of the chain (Borek, 1963; Gordon & Boman, 1964; Gordon, Boman & Isaksson, 1964), measure of methylated and total RNA after periods of labelling and chasing might furnish a direct proof of turnover without use of actinomycin D.

Exponentially multiplying organisms were incubated with single and both virginia-mycins for different periods, labelled for 8 min. with [^{14}C]methyl-L-methionine (as

methyl group donor) and [³H]uracil (as total RNA marker), and either extracted or chased with an excess of unlabelled methionine and uracil. RNA was fractionated by ultracentrifugation, and the acid-insoluble ^{14}C and ^{3}H radioactivities of single fractions were measured.

Ultracentrifugal patterns of methylated and total RNA from labelled bacteria are shown in Fig. 9A, B and C. Control samples show two well-defined peaks of ^{14}C and ^{3}H radioactivities overlapping the 23S and 16S absorbance peaks of rRNA, and no evident sign of turnover upon chasing. In bacteria incubated for a few minutes with either virginiamycin, little or no 23S RNA was methylated nor did it appear to be ^{3}H-labelled (Fig. 9B). Upon chasing—still in the presence of virginiamycin—some ^{14}C radioactivity was released from the rRNA peaks and could be recovered in the low molecular weight region (Fig. 9C).

Table 3. *Metabolism of methylated RNA in the presence of virginiamycins*

Experimental conditions as in the legend for Fig. 9. Samples within each RNA peak were pooled according to the scheme of Fig. 6A. ^{14}C and ^{3}H TCA-insoluble radioactivity was measured.

Virginia-mycins (µg./ml.)	Labelling* (min.)	Chasing (min.)	TCA-insol. cpm × 10⁻³ [¹⁴C]methyl-L-methionine in RNA					Ratio ¹⁴C/³H in rRNA
			23S	16S	10S	4S	< 4S	
—		—	4·12	5·48	2·25	6·87	8·06	0·380
		10–20	5·02	7·20	1·96	6·78	6·53	0·372
M 50		—	3·14	3·37	1·98	7·11	3·95	0·186
	2–10	10–20	1·01	1·38	1·34	9·07	2·50	0·144
S 50		—	3·34	3·91	2·90	11·04	1·41	0·187
		10–20	1·33	2·36	1·87	10·08	1·03	0·176
M+S 5		—	3·08	3·98	2·91	8·81	2·28	0·158
		10–20	3·46	2·80	2·74	6·37	2·72	0·163
—		—	4·67	4·90	2·45	7·81	4·40	0·313
		23–33	6·47	7·32	3·11	13·05	4·37	0·301
M 50		—	1·12	1·05	0·74	4·64	0·80	0·143
	15–23	23–33	1·14	0·84	0·90	6·48	1·35	0·167
S 50		—	1·36	1·56	1·03	4·75	0·91	0·161
		23–33	1·07	2·58	1·36	7·29	1·24	0·235
M+S 5		—	1·32	1·16	0·72	4·42	0·17	0·110
		23–33	0·77	0·55	0·61	4·05	4·05	0·123

* [¹⁴C]methyl-L-methionine and [6-³H]uracil.

Samples within each RNA peak were pooled according to the scheme of Fig. 6A, ^{14}C and ^{3}H radioactivities of each fraction were measured, and the results are tabulated in Table 3. It is clear that the rRNA which was methylated in the presence of virginiamycins underwent turnover upon chasing, an effect that was quite pronounced after short treatment with single virginiamycins. The inhibition of RNA synthesis occurring after prolonged incubation with the antibiotic obscured turnover at later times.

Thus rRNA (essentially 16S) which was made in the presence of virginiamycin decayed, yielding low molecular weight fragments which could be re-incorporated with high efficiency into rRNA in the presence of virginiamycin. In addition, the degree of methylation of the rRNA which was made in the presence of virginiamycin was lower than that of the controls, as indicated by its $^{14}C/^{3}H$ ratio (Table 3).

DISCUSSION

A mixture the two virgimamycins increases by 100-fold the antibiotic activity of the separate factors and has a bactericidal effect on *Bacillus subtilis*. Similar effects were observed with streptogramin (Chabbert & Acar, 1964), pristinamycin (Murat & Pellerat, 1965), and ostreogrycin (Vazquez, 1966a) in different micro-organisms. The irreversible loss of viability is preceded by a reversible alteration consisting of a long lag in colony formation on nutrient agar. A similar phenomenon was described by others and called 'bacteriopause' (Videau, 1965). This two-step effect can be explained by data which are presented in another paper of this series, which shows that virginia-mycin components bind to cell structures engaged in protein formation. Conceivably, the block of a sufficiently great number of such units may cause an irreversible loss of viability, whereas the inactivation of a smaller proportion of them might allow some growth at slower rate, which causes the inhibitor to be 'diluted out' during subsequent generations. Also, the synergistic antibiotic activity of virginiamycin components can be explained by their common target and complementary action on protein synthesis.

Inhibition of protein formation by virginiamycin may be the result of a block of either transcription or translation: in the first case synthesis of both RNA and proteins will be prevented, whereas in the second case only RNA would be synthesized. Data presented in this paper show that short periods of incubation with virginiamycin, which are able to stop amino acid incorporation completely, have little or no effect on the formation of polynucleotide chains: a block of transcription can, thus, be ruled out. Such a conclusion, which agrees with previous reports on ostreogrycins (Yamaguchi & Tanaka, 1964; Vazquez, 1966a), has received further confirmation by a study of virginiamycin action in *Bacillus subtilis* infected with virus 2C. In this system, virginiamycin specifically blocks the synthesis of nucleic acid which depends on an active protein formation (Cocito, 1969).

Data of Table 2 and Fig. 5 show that formation of ribosomal RNA is stimulated by short incubation with virginiamycins and inhibited after complete block of protein synthesis. Similar observations were made by Kurland & Maaløe (1962) in *Escherichia coli* treated with chloramphenicol. These authors conclude that proteins are required for rRNA formation, not for tRNA synthesis, as well as for the regulation of the balance between RNA species. Data presented in this work are in very good agreement with their hypothesis.

Figure 7 shows that virginiamycins interfere with the decay of pulse-labelled RNA. Decay of mRNA does not depend on protein synthesis (since it occurs in the absence of polypeptide chain formation: Fan, Higa & Levinthal, 1964), but on the incorporation of messenger into, and its release from, the polysomes. The joint inhibition of messenger decay and protein synthesis by virginiamycin could then be explained by a block of translation at the polysome level, an interpretation that will be proved in a further paper (C. Cocito, to be published).

The usual labelling-chasing techniques have shown a limited turnover of the rRNA which is synthesized in the presence of chloramphenicol, presumably because of the high efficiency of re-utilization of breakdown products of decayed polynucleotides (Neidhardt & Gros, 1957). To circumvent this difficulty, Fan et al. (1964) and Nakada (1965) employed actinomycin D: they showed that chloramphenicol- and puromycin-RNAs are degraded to low molecular weight material when *de novo* RNA synthesis is prevented. Conclusions drawn by the combined use of inhibitors of transcription

and translation can be criticized, however; consequently, in the present work, use was made of a double labelling technique (Dubin & Elkort, 1965). Since unmethylated rRNA is likely to be synthesized during transcription, re-incorporation of a methylated fragment might not occur at all, or might occur with lesser efficiency than an unmethylated one. Data reported in Table 3 and Fig. 8 furnish a direct demonstration that the rRNA which is made in the presence of virginiamycin is unstable and undergoes turnover.

[^{14}C]methyl group tracing in the control samples (Fig. 8) shows a higher peak in the 16S than in the 23S region. This is in agreement with the finding that 16S RNA contains 20 % more methylated bases than 23S RNA (Gordon & Boman, 1964).

'Relaxed' and 'chloramphenicol' particles contain undermethylated rRNA and tRNA: for example, under threonine starvation the level of rRNA methylated is 60 % and that of tRNA 90 % of the control (Gordon *et al.* 1964). A similar conclusion can be drawn for virginiamycin. Since the stability of rRNA apparently depends on the degree of methylation, undermethylated rRNA is expected to be unstable and undergo turnover Moreover, since mRNA, unlike rRNA, is not methylated, the hypothesis can be formulated that RNA function also depends on the degree of methylation (see Otaka, Osawa & Sibatani, 1964) and will be altered by virginiamycin.

Finally, the unstable RNA fraction indicated in this work as 'pulse-labelled' RNA presumably includes messenger RNA and decaying rRNA: virginiamycins increase the half-life of such 'pulse-labelled' RNA by inhibiting the decay of RNAs capable of a messenger function, and by rendering unstable the RNAs which are endowed with metabolic stability under physiological conditions. Similar observations were made by others with the RNA produced during methionine starvation by 'relaxed' mutants of *Escherichia coli* (Nakada, Anderson & Magasanik, 1964).

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The Action of Virginiamycin
on Nucleic Acid and Protein Synthesis in *Bacillus subtilis* Infected with Bacteriophage 2C

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SUMMARY

Addition of virginiamycin M or S before the virulent phage 2C prevented *Bacillus subtilis* from lysing, and this effect was increased 100-fold by simultaneous addition of both factors. When the antibiotics were administered at the end of the eclipse phase, the viral cycle was shortened by virginiamycin S, prolonged by factor M and halted by the two inhibitors together. Virginiamycins also prevented the accumulation of phage particles by inhibiting the synthesis of viral precursors during the eclipse period as well as during the maturation phase.

Synthesis of cellular macromolecules was decreased (not suppressed) in *Bacillus subtilis* after infection with phage 2C, and the degree of repression was a function of the input multiplicity. Viral inhibition of thymidine and uridine incorporation into host DNA and RNA was prevented when virginiamycin was added before infection but unaffected when addition was made at the end of the eclipse phase. Virus and virginiamycin had additive non-overlapping effects on protein synthesis. Moreover, virginiamycins interfered with the function, not the formation, of RNA pulse-labelled after infection, and prevented its decay.

It can be concluded that virginiamycin blocks (*a*) the preferential translation of viral message, (*b*) the mechanism by which virus halts host-macromolecule formation, and (*c*) the synthesis of viral DNA. This can be explained by an inhibitory action of virginiamycin on the synthesis and function of virus-dictated proteins.

INTRODUCTION

Virginiamycin, an inhibitor of mycotic origin, contains two factors, M and S (Vanderhaeghe, van Dijck, Parmentier & De Somer, 1957; Vanderhaeghe & Parmentier, 1960), which have a synergistic action in the sensitive bacterial strains. In the accompanying paper (Cocito, 1969) evidence is presented that virginiamycins block protein biosynthesis in *Bacillus subtilis* by interfering with polypeptide chain formation at the level of translation. The process of transcription is not inhibited by the antibiotic, the alterations observed in the metabolism of nucleic acids being due to interference with the process of protein formation.

The aim of the present investigation was to gather additional information about the mechanism of virginiamycin activity in bacteria. Virus-infected cells, in which numerous metabolic patterns are blocked and some others magnified, offer obvious advantages for this type of study. The system used, that of the DNA-containing

bacteriophage 2C and its host *Bacillus subtilis*, bears some peculiar biochemical features, which will be described elsewhere: unlike the well-known *Escherichia coli* T2 system (see Cohen, 1963), but similar to *E. coli* C infected with phage ϕX174 (Ruckert & Zillig, 1962) *Bacillus subtilis* infected with the virulent phage 2C continues forming, though at decreased rate, most of its macromolecules during the first 13 min. of the cycle (eclipse phase). Such biosyntheses become progressively decreased as the maturation progresses and progeny viral DNA accumulates. The onset of lysis (40 to 60 min. after infection) and the degree of inhibition of host macromolecule formation depend on the multiplicity of infection, a situation comparable to that recently described in *Shigella dysenteriae* infected with phage T4 (Terzi, 1967).

METHODS

Abbreviations used: HMU = hydroxymethyluracil; p.f.u. = plaque forming units. Additional abbreviations are listed in the accompanying paper (Cocito, 1969).

Host cells. The following strains of *Bacillus subtilis* were used: (1) 168/6, wild type; (2) 168/2, leu⁻, trp⁻; (3) A26, U⁻, trp⁻; (4) W23, T⁻, leu⁻, trp⁻; (5) 6633, wild type. *Bacillus subtilis* was plated on L agar at 37° for viable counts; for microscope counts infected bacteria were fixed and stained in a suitable dilution with a solution 5% iodine in 10% (w/v) KI.

The composition of the media used for growing stocks and labelling cells is reported in the accompanying paper (Cocito, 1969).

Virus production, purification and titration. Routinely, phage stocks were produce either on 168/2 or on A26. Bacteria growing exponentially in L medium were infected with 1 to 5×10^8 particles/10^8 bacteria/ml. Cultures were shaken at 37° until lysis occurred. Lysates were centrifuged at 9000 g for 15 min. to remove cell debris, and phage particles were sedimented for 30 min. at 15,000 g. Particles were suspended either in 0·15 M-NaCl buffered at pH 7·4 with 0·01 M-tris and containing 40 μg. serum albumin/ml. (^{32}P experiment), or in the same solution with 0·05 M-phosphate buffer (pH 7·4) replacing the tris (^{3}H and ^{14}C experiments). Concentrated phage was purified through two cycles of low- and high-speed centrifugations, followed by centrifugation for 32 hr at 33,000 rev./min. at 12° in CsCl ($\rho_{25°}$ = 1·52 g./ml.) using the SW39 rotor of a Spinco preparative centrifuge. The band of virus was collected and stored at 4° after addition of 1 to 5% (v/v) of dimethylsulphoxide (Yehle & Doi, 1965). Exponentially growing bacteria were used as indicator strains for titrating phage by plaque assay. Taking the number of plaques in 168/2 as 100, the relative efficiency of plating was on the average 65% for A26 and 75% for 168/6.

Disruption of infected host bacteria. Titration of intracellular virus was carried out on enzymic lysates of host bacteria. Metabolism was stopped by transferring samples of the suspension to an ice bath and adding KCN (0·01 M). After addition of 10 μg. lysozyme/ml., the samples were incubated for 20 min. at 37°, and then diluted with ice cold DSP (0·15 M-NaCl, 0·01 M-K phosphate buffer (pH 7·5) containing 0·5 mg. peptone/ml.). Other procedures for release of infectious intracellular virus, such as lysis with lysozyme in the presence of chloroform (1/10, v/v) and disruption in a French press of bacteria suspended in 0·01 M-NaN₃, had lower efficiency (15 to 75%) as compared to the KCN+lysozyme procedure.

For isolation of nucleic acids, bacteria were collected and disrupted in a French

press as detailed by Cocito (1969). Bacteria were suspended in 0·1 M-NaCl+0·1 M-tris buffer (pH 7·4) before disruption, and then treated with electrophoretically pure DNAse (1 to 5 μg./ml.) at 4° to 23° for 5 to 10 min. Enzymic reaction was stopped by addition of 0·5 % (w/v) SDS.

Biochemical and biophysical determinations. Methods for colorimetric, radioactivity and spectrophotometric determinations, and procedures for extraction, fractionation and measurement of nucleic acids, were described by Cocito (1969), where preparation of crystalline virginiamycins and the source of chemicals is also given.

RESULTS

Action of virginiamycin on growth and lysis of virus-infected bacteria. Exponentially growing cultures of the sensitive *Bacillus subtilis* strains (168/2, A26, 168/6) lysed 40 to 6c min. after infection with the virulent bacteriophage 2C. The length of the latent period was inversely proportional to the multiplicity of infection and the generation time. Turbidimetric tracings indicated that bacteria infected with 1 to
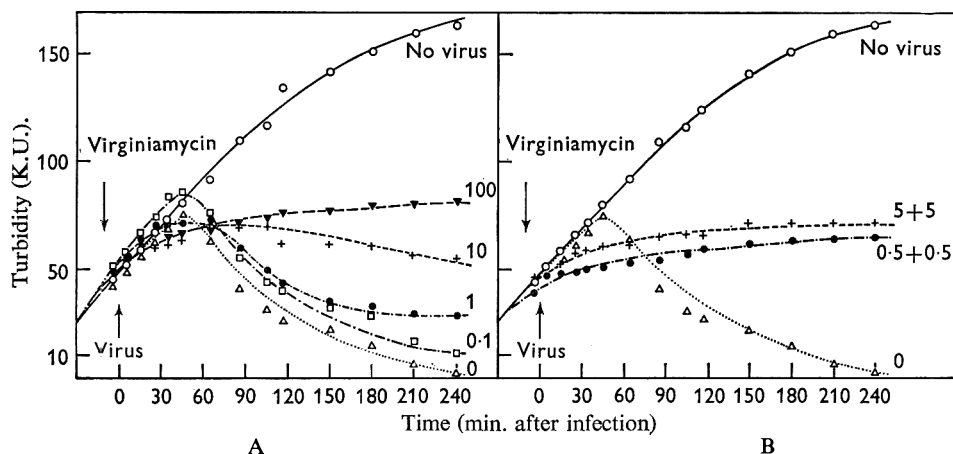

Fig. 1. Growth and lysis of virus-infected Bacillus subtilis in the presence of increasing amounts of virginiamycin. Strain: 168/2. Medium: OS, supplemented with 5 mg. Glc, 4 μg. Trp, 8 μg. Leu and 800 μg. ECD/ml. Infection: input multiplicity of 10 p.f.u. of CsCl-purified virus, added at time 0 to all the cultures but one (control —O—: no virus, no inhibitors). Virginiamycin (A) = factor S (0, 0·1, 1, 10, and 100 μg./ml.); (B) = both factors (0·5 and 5·0 μg./ml.) added 10 min. before the virus. Growth of the agitated cultures followed turbidimetrically (K.U.).

10 p.f.u. of virus 2C/bacterium continued growing at the same rate as the controls for the entire eclipse phase (microscope counts of fixed bacteria showed an increase in number). During the maturation phase growth was progressively decreased and then halted; lysis occurred after a stationary phase of about 10 min. and was complete.

Increasing amounts of virginiamycins, either separately or together, were added to exponentially growing cultures of *Bacillus subtilis* 168/2. Bacteria were infected 2 min. later with low multiplicity (10 p.f.u./bacterium) of virus 2C, and the turbidity of the cultures was followed for several generation times. As the concentration of single virginiamycins was increased, the length of the latent period increased; a quite high concentration of the inhibitor was required, however, to block lysis com-

pletely (Fig. 1A). Factors M and S together increased their activity by more than 100-fold since lysis inhibition, which could be obtained with 100 μg. of single virginiamycins, occurred equally with 0·5 μg. of their mixture (Fig. 1 B).

In further experiments virginiamycins were added either before or after the eclipse phase to cultures of *Bacillus subtilis* 168/6 infected at high multiplicity (50 to 150 p.f.u./ bacterium). Under these conditions early and rapid lysis occurred in the controls, whereas lysis was prevented by addition of virginiamycins at the time of infection (Fig. 2A). When virginiamycin was added at the end of the eclipse period, the latent period was shortened by virginiamycin S, and prolonged by factor M, whereas the simultaneous addition of both components still prevented lysis completely (Fig. 2B).
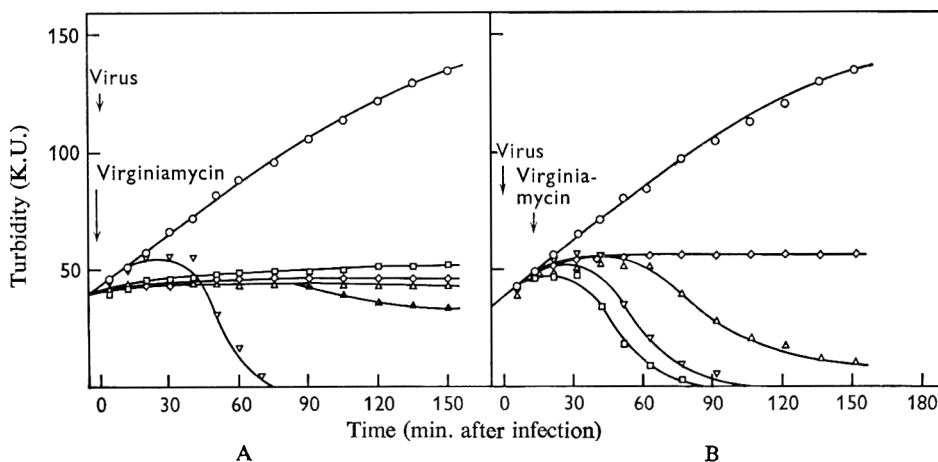

Fig. 2. Relationship between time of addition of virginiamycin and lysis of virus-infected bacteria. Strain: *Bacillus subtilis* 168/6. Medium: OS, supplemented with 5 mg. Glc, and 800 μg. ECD/ml. Infection with 50 p.f.u. of CsCl-purified virus/bacterium, added at tim 0 to all the cultures but one (control —O—: no virus, no inhibitors). (A): virginiamycin added 2 min. before the virus: none —∇— (control of infected cells); 50 μg. factor M —△—; 50 μg. factor S —□—; 5 μg. M+S/ml. —◇—. Sample —▲— same amount of virginia-mycin M, but infection with 150 p.f.u./bacterium. (B): virginiamycin (same concen-tration and symbols) added 15 min. after the virus.

These results indicate that either the synthesis or the function of a phage enzyme, on which lysis of infected organisms depends, was blocked by the simultaneous presence of both virginiamycins, but not by single components, during the maturation phase. However, factors M and S separately exerted a full inhibitory activity when their addition was made at the beginning of the eclipse phase.

Formation of viable phage particles in the presence of virginiamycin. Additional information can be obtained by titration of intracellular particles released by artificial lysis from virginiamycin-treated virus-infected bacteria (progeny particles start accumulating intracellularly about 15 min. after infection).

Data reported in Table 1 show that addition of single virginiamycins before infection halted phage replication: this effect paralleled lysis inhibition. Thus, con-centrations of factors M and S sufficient to decrease growth of control bacteria by more than 70 % prevented lysis of infected bacteria and stopped production of viral particles. When the level of one virginiamycin was insufficient to prevent lysis, there was only

limited production of progeny particles. Association of the two virginiamycins at very low concentrations blocked lysis of the host cells and virus formation. In addition, there was a progressive decrease of the number of infectious centres as the time of incubation with the drug increased.

Synthesis of viral DNA in virginiamycin-treated bacteria. In the absence of viable progeny particles, the question can be raised whether the formation of viral DNA is also blocked by the antibiotic. Phage 2C DNA contains HMU in the place of thymine, which allows viral DNA to be distinguished from host DNA synthesis.

Table 1. *Formation of viable phage particles in the presence of virginiamycin*

Strain, medium and input multiplicity as for Fig. 1. Virginiamycin added 10 min. before the virus. Samples diluted with ice-cold lysing fluid containing 100 μg. lysozyme and 0·01 M-KCN, incubated 30 min. at 4° and 20 min. at 37°, and further diluted in ice-cold DSP. Titration of released virus as outlined in materials and methods, by using *Bacillus subtilis* 168/2 as indicator strain.

Time of sampling (min. after infection)	Control (no phage; no virginiamycin)	Phage-infected bacteria						
		No virginiamycin	Factor M		Factor S		M+S	
			(5 μg.)*	(50 μg.)	(1 μg.)	(10 μg.)	(0·1+0·1)*	(1+1)
			Phage yield (× 10⁻⁸ p.f.u./ml.)†					
0–10§	—	2·1	1·6	2·8	2·1	2·0	2·2	1·9
10–35	—	11·0	5·4	4·6	5·3	4·0	6·1	4·7
35–45	—	17·0	3·3	—	3·8	4·5	5·9	5·0
45–55	—	23·0	4·2	2·5	2·5	2·1	4·3	2·3
55–65	—	36·0	8·3	2·8	2·9	1·7	4·7	2·1
65–75	—	48·0	9·5	—	5·0	—	3·6	—
75–95	—	50·0	—	2·5	9·5	—	3·0	—
95–125	—	—	13·0	—	9·5	1·1	—	1·5
125–21C	—	—	—	1·1	—	1·0	2·8	0·9
			Turbidity (K.U.₆₇₀ ₘμ)					
30	71	71	60	70	70	69	51	68
90	104	10	50	75	82	81	67	77
180	134	2	9	73	9	68	64	90

* μg. of single factors added to 1 ml. of medium 10 min. before the virus.

† Average number of plaques counted in samples taken within the indicated period.

§ Infectious centres titrated upon removal of unadsorbed virus by centrifugation 3 min. after infection.

Virginiamycins M and S singly and together were added to the cultures either before or after infection with a high multiplicity of phage 2C. The kinetics of incorporation of [³H]uridine into viral DNA was then followed during the viral cycle. Virginiamycins, either singly or together, inhibited the synthesis of viral nucleic acid, and a similar effect was obtained whether addition was made at the beginning (Fig. 3A) or at the end (Fig. 3B) of the eclipse period, when translation of early cistrons had already occurred.

Thus, both virginiamycins specifically block the formation of viral DNA catalyzed by hypothetical virus-specific DNA polymerases. Moreover, since addition of factor S at the end of the eclipse phase prevented viral DNA formation but not cell lysis, we may conclude that lysozyme could be made in the absence of an active synthesis of viral DNA.

Synthesis of host DNA in the presence of virginiamycin. Virginiamycins inhibit the formation of viral DNA: what is their action on host DNA synthesis in virus-infected bacteria? The problem was investigated by following the kinetics of incorporation of [³H]thymidine during the viral cycle (2C-DNA does not contain thymine). The inhibitors were added to the culture either before or at the end of the eclipse period.

Infection of strain 168/6 at high multiplicity (50 p.f.u./bacterium) inhibited the incorporation of thymidine into host DNA sharply. The degree of inhibition was at least 70% during the first 5 min. and 85% during the next 5 min. of the viral cycle, and the amount of thymidine incorporated during the eclipse phase into host cell DNA was no more than 10 to 15% that of control bacteria. Under these conditions addition of virginiamycins before the virus largely prevented the inhibition of host DNA formation: the effect of factor M lasted for the entire viral cycle, whereas that of factor S was limited to the eclipse phase (Fig. 4).
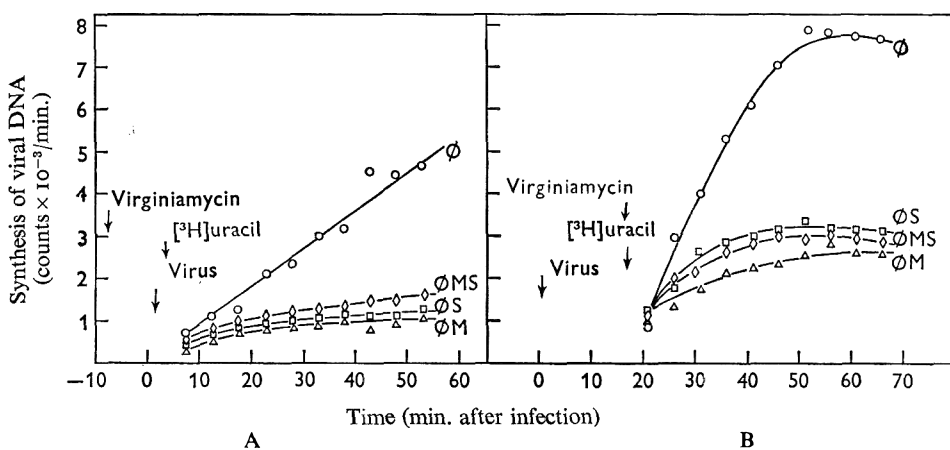

Fig. 3. Formation of viral DNA in the presence of virginiamycins. Strain, medium, and infection as for Fig. 2. (A): 50 μg. of single virginiamycins, or 2·5 μg. of both components added 8 min. before the virus. (B): same concentrations of inhibitors added 14 min. after the virus. Labelling: [6-³H]uracil, (specific activity 12 c/mmole, 1 μc/ml., f.c.), added either 2 min. after the virus (3A) or 2 min. after virginiamycin (3B). TCA-insoluble radioactivity, precipitated in the presence of 200 μg. unlabelled uracil and 100 μg. BSA/ml., collected on micropore filters. Membranes eluted with 0·5 M-KOH for 15 min. at 23° and counted again (to determine the amount of residual radioactivity). Eluates further incubated for 18 hr at 37° and alkali-resistant TCA-insoluble radioactivity (DNA) collected on micropore filters and counted (Cocito, 1969).

Incorporation of amino acids into polypeptides: action of infection and virginiamycin. Since, upon infection, the inhibition of host cell DNA preceded the formation of viral DNA, one may suppose that the latter was not responsible for the former. Inhibition of host cell DNA might be due, instead, to some virus-induced early proteins. If so, one would predict that infection affected protein synthesis far less than formation of host cell DNA, and that virus and virginiamycin will have additive non-overlapping effects on polypeptide chain formation.

This prediction was verified by studying the incorporation of [¹⁴C]amino acids by *Bacillus subtilis* infected with phage 2C and incubated in the presence of virginia-

mycins. Figure 5 shows that viral infection decreased the incorporation rate, and that the inhibitory effect was magnified when infection was carried out in the presence of virginiamycin. At the end of the eclipse period, for example, amino acid incorporation in virus-infected bacteria was about 10% that in uninfected bacteria when virginiamycin was present, and 70% when it was absent.

Comparison of Fig. 5 with data reported in the accompanying paper (Cocito, 1969) shows that the degree of inhibition of protein synthesis by virginiamycin in infected bacteria was about double that in uninfected bacteria. Hence, virus infection and virginiamycin repress protein synthesis through different mechanisms, and their effects are additive.
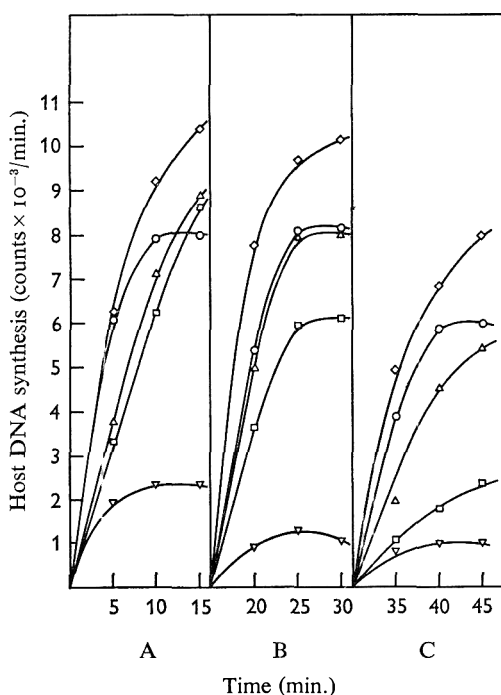

Fig. 4   Fig. 5

Fig. 4. Rate of labelling of host DNA in virginiamycin-treated virus-infected bacteria. Strain: *Bacillus subtilis* 168/6. Medium: OS, supplemented with 5 mg. Glc, 800 μg. ECD/ml. Virginiamycin: none (control of uninfected bacteria —O—; control of infected bacteria —▽—); 50 μg. factor M —△—; 50 μg. factor S —□—; 2·5 μg./ml. of both components —◇—, added to the cultures 9 min. before infection. Virus: 30 p.f.u./bacterium added 1 min. before labelling to all the cultures but one (control —O— of uninfected bacteria). Labelling: [³H]thymidine (specific activity 17 c/mmole, 0·104 μc/ml., f.c.) added at 1 (A), 16 (B) and 31 (C) min. after infection. Sampling: every 5 min. for 15 min. TCA-insoluble radioactivity collected on micropore filters in the presence of 100 μg. unlabelled thymidine and BSA/ml., and counted.
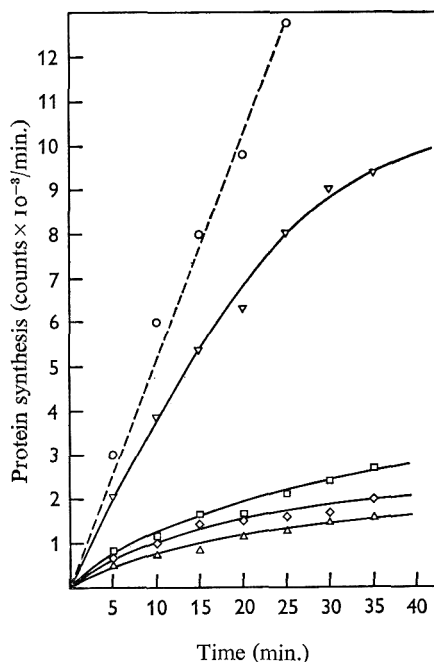
Fig. 5. Kinetics of incorporation of amino acids into proteins: action of virginiamycin and infection. Strain: *Bacillus subtilis* A26. Medium: OS, supplemented with 5 mg. Glc, 4 μg. Trp, 8 μg. Leu, 800 μg. ECD and 100 μg. U/ml. Infection: 30 p.f.u. of virus added 9 min. after virginiamycin and 1 min. before labelling to all the cultures but one. Virginiamycin: none (control of uninfected bacteria —O—; control of infected bacteria —▽—); 50 μg. factor M —△—; 25 μg. factor S —□—; 2·5 μg. M+S/ml. —◇—. Labelling: [¹⁴C]algal protein hydrolysate (specific activity 520·8 μc/mg., 0·417 μc/ml., f.c.) added at time 0. TCA-insoluble radioactivity collected on micropore filters and counted.

Kinetics of polynucleotide formation in virginiamycin-treated host cells. In uninfected bacteria virginiamycin represses polyribonucleotide and deoxyribonucleotide formation only after protein synthesis has been halted (Cocito, 1969). Since transcription and translation of early viral cistrons occur within a few minutes, it is logical to suppose that the virus would affect host RNA synthesis more rapidly and profoundly than do the virginiamycins. Data which follow show the correctness of this prediction.

Incorporation of labelled uracil into polyribonucleotides was followed in bacteria which were treated first with virginiamycins and then infected with virus 2C. Radio-
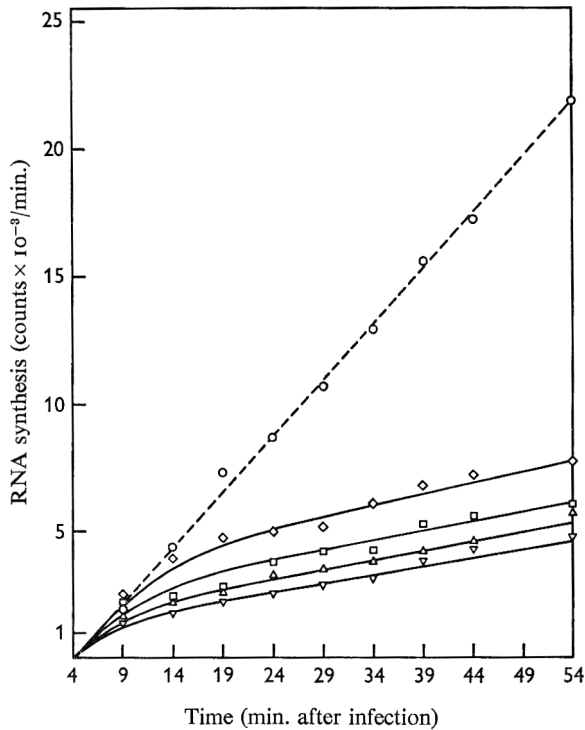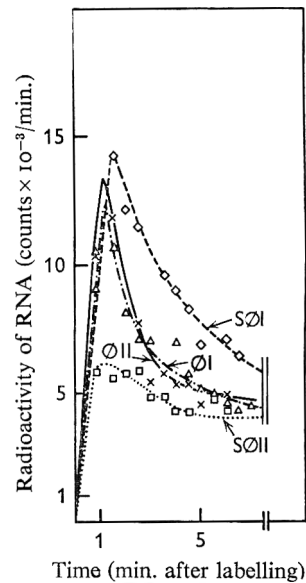
Fig. 6   Fig. 7

Fig. 6. Incorporation of uracil into polyribonucleotides, after infection with virus 2C in the presence of virginiamycins. Strain, medium and conditions of infection as for Fig. 5. Virginiamycins: none (controls of uninfected —O— and virus-infected —▽— bacteria); 50 μg. factor M (—△—), 25 μg. factor S (—□—), 2·5 μg. M+S (—◇—), added 4 min. before labelling. Bacteria infected with 30 p.f.u. of virus 2C/bacterium 4 min. before isotope addition. Labelling: [6-³H]uracil (specific activity 12 c/mmole, 0·835 μc/ml., f.c.) added at time o. TCA-insoluble radioactivity precipitated in the presence of 100 μg. of unlabelled uracil and 100 μg. BSA/ml., collected on micropore filters and counted. Values corrected for KOH-resistant radioactivity (see legend for Fig. 3 and Cocito, 1969).

Fig. 7. Decay of pulse-labelled RNA in virginiamycin-treated virus-infected bacteria. Strain and medium as for Fig. 1. Virus: 15 p.f.u./bacterium of CsCl-purified 2C added 1 min. before labelling. Virginiamycins: none (controls of virus-infected bacteria Ø1 and Ø2); 5 μg. factors M+S added 10 min. before the virus (SØ1 and SØ2). Labelling time (time o): [6-³H]uracil (specific activity 12 c/mmole, 3·58 μc/ml., f.c.) added 8 min. (Ø1 and SØ1) or 23 min. (Ø2 and SØ2) after infection. Chasing (at 45 sec.); 200 μg. of unlabelled uracil and 15 μg. of actinomycin D/ml. TCA-insoluble radioactivity precipitated in the presence of 200 μg. unlabelled uracil and 100 μg. BSA/ml., collected on micropore filters and counted.

activity determinations were corrected for label incorporated into viral DNA. Figure 6 shows that infection with phage 2C repressed incorporation of uracil very quickly. At the end of the eclipse phase the incorporation rate in infected bacteria was about one third that of uninfected bacteria. The inhibitory effect caused by the virus during the eclipse phase was reduced when infection was carried out in the presence of virginiamycin. This antagonistic action could not be demonstrated during the maturation phase, however, because the blockage of RNA synthesis due to the virginiamycin-mediated halt of protein synthesis (see Cocito, 1969) obscured the whole picture.

Synthesis and decay of pulse-labelled RNA in virus-infected bacteria incubated with virginiamycin. Data reported in the previous sections support the idea that virginiamycins might prevent formation of virus components and block virus-specific biochemical reactions by halting the synthesis and function of virus-dictated proteins. A study of mRNA metabolism would be an approach to this problem.

Virus-infected bacteria were pulse-labelled with [^{3}H]uracil in the presence of virginiamycin, and chased 45 sec. later with an excess of unlabelled uracil and actinomycin. The use of this inhibitor of transcription for preventing re-incorporation of chased radioactivity in uninfected virginiamycin-treated *Bacillus subtilis* has been stressed already (Cocito, 1969). Samples of labelled and chased cultures were withdrawn at different times, and the radioactivity incorporated into polyribonucleotides was measured.

Figure 7 shows that virginiamycins interfered with the decay of pulse-labelled RNA, with a consequent increase of the half-life of this RNA. Under our experimental conditions average values of 150 and 240 sec. were calculated for the half-lives of early and late messenger viral RNAs (compare these figures with those for host messenger RNA, for which an average value of 90 sec. (Cocito, 1969) was reported). It is difficult to calculate exactly the half-life of pulse-RNA in virginiamycin-treated virus-infected bacteria; values of 7 to 12 min. were obtained.

It can be concluded that virginiamycins M and S interfere with the function, not with the synthesis, of pulse-labelled RNA. These findings exclude a block of transcription and predict an alteration of the translation mechanism.

DISCUSSION

Lysis inhibition of bacteria infected in the presence of virginiamycin is probably due to a blockage of phage enzyme formation. Such an effect can be accounted for by the rapidity with which the antibiotic halts the incorporation of amino acids into polypeptides (see Fig. 5 and Cocito, 1969). Addition of virginiymycin M or S at the end of the eclipse phase still prevents the synthesis of viral components, not the lysis of the host cell (Fig. 2B). The simplest explanation is that mRNA for lysozyme formation is made during the entire viral cycle, its concentration at any given moment being proportional to that of viral template. If so, the amount of lysozyme present at the end of the eclipse phase would be too small for a precise biochemical evaluation (Pene & Marmur, 1967), yet sufficient to cause the lysis of the host cell at a reduced rate (Fig. 2B). Such interpretation of data reported in the present work is in perfect agreement with the conclusions drawn by Edlin (1965a, b) with the T4 *Escherichia coli* system.

The observation that virginiamycin, when added before virus 2C, prevents the synthesis of viral precursors, parallels earlier findings with chloramphenicol-treated T2-infected *Escherichia coli* (Tomizawa & Sunakawa, 1956; Hershey & Melechen, 1957). And this is probably true for all inhibitors of protein synthesis, whether active at the transcriptional or translational level (Cohen, Sekiguchi, Stern & Barner, 1963; Cohen, 1963): when there is a blockage in the translation of the mRNA corresponding to the early cistrons, formation of the enzymes necessary for viral DNA synthesis is prevented. The simplest explanation for the inhibition of viral DNA which occurs when virginiamycins is added at the end of the eclipse phase is that the drug interferes with the function of virus-specific DNA polymerases which are already made by that time. Since the synthesis of host DNA, unlike that of viral DNA, is not altered by virginiamycin (Cocito, 1969), one may predict that the antibiotic has completely different actions on host and viral DNA-polymerases.

In *Bacillus subtilis* infected with virus 2C the synthesis of host RNA and proteins is only partly repressed, a situation comparable to those recently observed in *Shigella dysenteriae* infected with phage T4 (Terzi, 1967), and *Escherichia coli* C infected with the 'clear' non-lysogenizing mutant of λ (Terzi & Levinthal, 1967). The main difference between λc-infected *E. coli* and 2C-infected *B. subtilis* is that, in the former case, a treatment with chloramphenicol before infection, though incapable of preventing the virus-mediated inhibition of uracil incorporation into RNA, suppressed completely amino acid incorporation into proteins (Terzi & Levinthal, 1967). Conversely, in the latter system, a level of virginiamycin capable of reducing the inhibition of RNA formation due to the virus (Fig. 6) increased still further the repression of protein synthesis caused by infection (Fig. 5). However, such differences between the two virus-host systems may not be real, because the experiments were performed with different levels of inhibitors.

Treatment of *Escherichia coli* with chloramphenicol before infection with phage T4 induces the following alterations of nucleic acid metabolism: (1) rRNA, tRNA and host DNA are synthesized, though at reduced rates with respect to uninfected controls (Nomura, Hall & Spiegelman, 1960; Nomura, Okamoto & Asano, 1962; Nomura, Matsubara, Okamoto & Fujimura, 1962); (2) the greater the multiplicity of infection, the higher the degree of inhibition of the host DNA fraction which is formed in the presence of the antibiotic; (3) for every multiplicity, RNA synthesis is inhibited to a greater extent than synthesis of host DNA (Nomura, Witten, Mantei & Echols, 1966). Some of the results reported in the present paper are similar to those with T4-infected, chloramphenicol-treated *Escherichia coli*. Virginiamycin added before the virus reduces the inhibition of host DNA synthesis to a greater extent than that of RNA formation (Fig. 4 and 6); this effect does not take place when the antibiotic is added at the end of the eclipse phase.

Kölsh (1965) reported that the synthesis of *Escherichia coli* DNA is not blocked after infection with u.v.-irradiated phage T2: thus, transcription and perhaps translation (Sköld & Buchanan, 1964; Furth & Pizer, 1966) of some viral cistrons are responsible for the inhibition of host chromosome replication. The work of Nomura *et al.* (1966) indicates that the T4-mediated inhibition of the synthesis of *E. coli* DNA may be determined as much by a chloramphenicol-sensitive virus-dictated protein as by an unknown chloramphenicol-insensitive mechanism related to the multiplicity of infection. My data agree with these conclusions and, in addition, exclude the possibility

that replication of viral DNA *per se* might be responsible for the inhibition of host-DNA, since the formation of the latter is still blocked under conditions which prevent the synthesis of 2 C-chromosome.

The half-life of the RNA which is pulse-labelled in bacteria infected in the presence of virginiamycin is prolonged. Similar observations have been made with uninfected bacteria after treatment with the antibiotic (Cocito, 1969). This indicates that virginiamycin interferes with the function, rather than the synthesis, of pulse-labelled RNA, and that the mechanism of action of the antibiotic is similar in uninfected and virus-infected bacteria.

The over-all conclusion is that virginiamycin interferes, not with transcription, but with the translation mechanism of both host and viral mRNA. Some 'early' viral cistrons apparently code for inhibitors (probably proteins) which repress replication and transcription of the host chromosome: they are responsible for the preferential reading of viral message which can be observed in the absence of antibiotics. Virginiamycins prevent the translation of the early viral message, and so mediate a derepression of host macromolecule formation. In addition, virginiamycins specifically inhibit the action of two or more virus-specific enzymes, thus preventing the formation of viable viral particles and their release by lysis of the host cell.

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The Function of the β-Ketoadipate Pathway in *Pseudomonas acidovorans*

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SUMMARY

Aerobic enrichment at 30° in a medium containing either *cis,cis-* or *cis,trans-*muconic acid as the sole source of carbon and energy is a highly specific method for the isolation of *Pseudomonas acidovorans* from soil. Nutritional studies with previously isolated strains of *P. acidovorans* and *P. testosteroni*, none of which was selected for the ability to utilize muconates, show that nearly all strains of both these species grow readily and promptly with both *cis* isomers of muconic acid. This ability is absent from all fluorescent pseudomonads examined, despite the fact that they possess the requisite enzymic machinery. The fluorescent pseudomonads appear to be impermeable to the muconic acids, and can use them as substrates for growth only through a mutation that alters the permeability of the cell.

Studies with one strain of *Pseudomonas acidovorans* show that it synthesizes inducibly all the enzymes responsible for the conversion of catechol to β-ketoadipate, their synthesis being elicited by growth with either *cis,cis-* or *cis,trans-*muconate. Mutants of *P. acidovorans* defective in the synthesis of either β-ketoadipate enol-lactone hydrolase or β-ketoadipate succinyl-CoA transferase are unable to grow at the expense of *cis,cis-*or *cis,trans-*muconate; this confirms the role of the β-ketoadipate pathway in the dissimilation of these two substrates by *P. acidovorans*.

INTRODUCTION

Aerobic bacteria belonging to many taxonomic groups dissimilate such aromatic and hydroaromatic compounds as benzoic acid, *p*-hydroxybenzoic acid, phenol, tryptophan, quinic acid and shikimic acid through one of the convergent branches of the β-ketoadipate pathway (Fig. 1). The β-ketoadipate pathway is the commonest, but not the sole, biochemical route for the oxidation of such primary substrates. In the genus *Pseudomonas*, where this pathway is used by most species capable of oxidizing aromatic and hydroaromatic compounds, two species—*Pseudomonas acidovorans* and *P. testosteroni*—dissimilate these compounds through entirely different metabolic pathways (Stanier, Hayaishi & Tsuchida, 1951; Stanier, Palleroni & Doudoroff, 1966; Wheelis, Palleroni & Stanier, 1967). Since the β-ketoadipate pathway is not known to operate in the dissimilation of any other class of primary substrates, it appeared to be absent from *P. acidovorans* and *P. testosteroni*. We have now found that this assumption is incorrect. *P. acidovorans* can synthesize inducibly many of the

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enzymes in the pathway, which function in the dissimilation of muconic acids by this species.

Cis,cis-muconic acid is an intermediate in the catechol branch of the β-ketoadipate pathway (Fig. 1). However, bacteria that use the β-ketoadipate pathway are characteristically impermeable to *cis,cis*-muconic acid, being unable either to respire it or
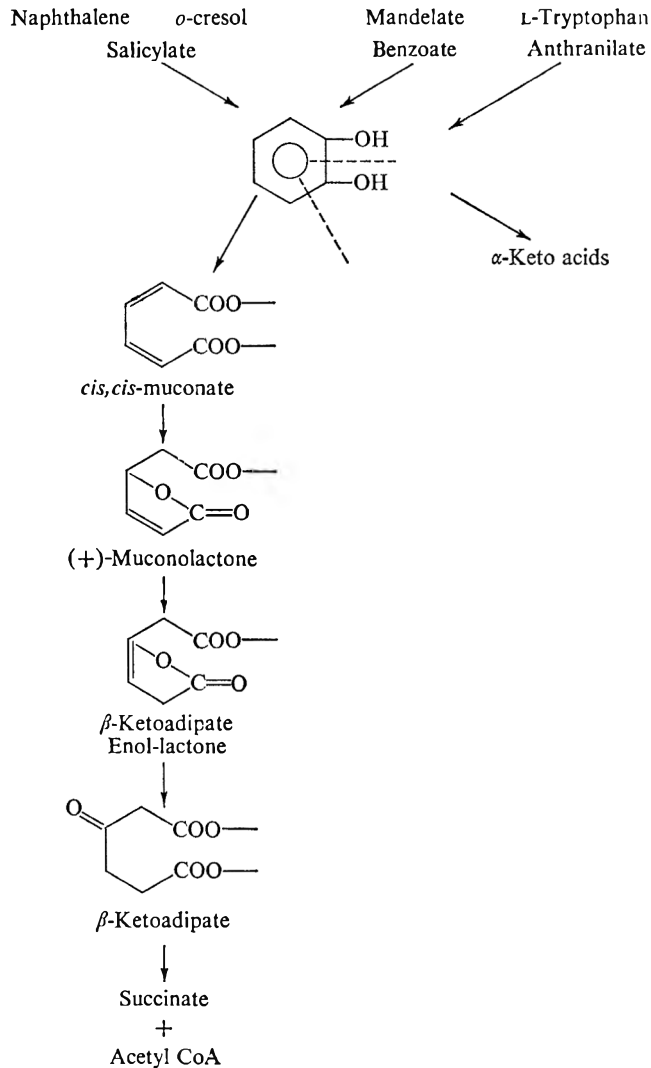
Fig. 1. Metabolic map showing dissimilation of aromatic compounds via catechol. Cleavage of catechol in *meta* position leads to α-keto acids; *ortho* cleavage of cathechol gives rise to the β-ketoadipate pathway.

to use it for growth. In *Pseudomonas putida* (Ornston, 1966*b*), *P. aeruginosa* (Kemp & Hegeman, 1968) and *Acinetobacter* (*Moraxella*) *calcoacetica* (Cánovas & Stanier, 1967) the barrier to its use can be overcome by mutation: selection for the ability to grow with *cis,cis*-muconate yields rare spontaneous mutants with greatly increased

permeabil:ty to this compound. The *cis,trans* isomer of muconic acid, which can be readily formed chemically from the *cis,cis* isomer (Elvidge, Linstead, Sims & Orkin, 1950 *a*), is not known to have a metabolic role. However, Sistrom & Stanier (1954) showed that it could be slowly converted to (−)-muconolactone by the muconate lactonizing enzyme of *P. putida*, which catalyses the reversible conversion of the *cis, cis* isomer to (+)-muconolactone.

The experiments described here originated from an attempt to ascertain the nature of the micro-organisms responsible for the dissimilation of muconic acids in Nature.

METHODS

Media and conditions of cultivation. All media were prepared from a basal salt solution of the following composition (g./l.): NH_4Cl, 1·0; KH_2PO_4, 2·72; Na_2HPO_4, 2·82; $MgSO_4$, 0·2; $CaCl_2$, 0·05; $FeSO_4 . 7H_2O$, 0·005; $Na_2MoO_4 . 2H_2O$, 0·001; $MnSO_4$, 0·001; $Co(NO_3)_2 . 6H_2O$, 0·001. The organic compound serving as source of carbon and energy was added from a concentrated, sterile stock solution, to 0·1 % (w/v). The final pH was 6·8. *cis,cis*-Muconate and other thermolabile substrates were sterilized by filtration. Solid media were prepared by separately sterilizing double strength mineral base and 2% (w/v) Ionagar No. 2, which were combined and supplemented with the carbon source after cooling to below 50°.

Stock cultures were maintained on succinate agar slopes. Liquid cultures were grown in flasks containing approximately 0·25 vol. medium, subjected to mechanical agitation, at 30°. Growth was determined turbidometrically in a Beckman model DK-2A spectrophotometer at 660 mμ.

Enrichment experiments. Two series of shallow layers of liquid basal medium, furnished respectively with *cis,cis*- or *cis,trans*-muconic acid as sole carbon source, were inoculated with 1 g. of five different soil samples and incubated at 30° without agitation. After 2 or 3 days, transfers were made to fresh media of similar compositions. Isolations were made by plating on the homologous agar medium, and the predominant bacterial type from each enrichment was purified and characterized by the methods described by Stanier *et al.* (1966).

Isolation of mutants. Mutants of *Pseudomonas acidovorans* strain 13 unable to grow with muconate were prepared by treatment with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, using the techniques of mutagenic treatment and selection described by Ornston (1966*b*).

Preparation of cell-free extracts. Exponentially growing organisms were harvested from 500 ml. of medium by centrifugation at 7000 *g* for 15 min. at 0°. The bacteria were washed by centrifugation in 20 mM tris-HCl buffer (pH 8·0) which was 10 μM in Mg-EDTA, and the pellets were resuspended in 5 ml. of the same buffer and stored at −20°.

Frozen suspensions were thawed and ultrasonically disrupted (10 min. with an MSE sonic disintegrator). The probe was pre-chilled and the samples were cooled in ice during treatment. The extract was centrifuged at 100,000 *g* for 45 min. in a Martin Christ 'Omikron' refrigerated ultracentrifuge. The supernatant fluid was used as crude extract for measurement of enzyme activities. The extracts were kept in cracked ice until all the enzymes had been assayed. Extractions and assays were generally performed on the same day.

Enzyme assays. The following enzymes were assayed by the methods of Ornston (1966a): catechol oxygenase (E.C. 1.99.2.2; catechol 1,2 oxygenase); muconate lactonizing enzyme (E.C. 5.5.1.1; 4-carboxy-methyl-4-hydroxyisocrotonolactone lyase (decyclizing), recommended trivial name 'muconate cycloisomerase'); (+)-muconolactone isomerase and β-ketoadipate enol-lactone hydrolase (no E.C. names). Transferase (E.C. 2.8.3.6; β-ketoadipate succinyl-CoA transferase or 3-oxoadipate-CoA transferase) activity was determined by the method of Cánovas & Stanier (1967).

All assays were performed at room temperature with a Beckman DK-2A recording spectrophotometer. With the exception of transferase activities, the unit of enzyme activity is defined as the amount of enzyme necessary to cause the disappearence of 1 μmole of substrate per min. under the conditions of assay employed. Since the extinction coefficient of the product measured in transferase assays (β-ketoadipyl-CoA) is not known, the unit of transferase activity is defined in arbitrary terms as the amount of enzyme necessary to cause a change of 1·0 (optical density) units per min. under the prescribed assay conditions.

Manometric experiments. Carbon dioxide evolution and oxygen consumption were measured in a Warburg apparatus (Braun, Model W85) at 30°. Exponentially growing bacteria were centrifuged and resuspended in 20 mM-phosphate buffer (pH 7) at a concentration of approximately 300 μg. dry weight/ml. The main compartment of each flask contained 1·6 ml. suspension; the centre well 0·2 ml. of KOH 20% (w/v); and the side-arm 5 μmole substrate. All data were corrected for endogenous oxygen uptake. β-Ketoadipate was quantitatively determined by catalytic decarboxylation with 4-amino-antipyrine as described by Sistrom & Stanier (1953).

Chemical measurements. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with a bovine serum albumin (purchased from NBC Research Biochemicals) as standard. Lactone was determined by the hydroxamate method of Hestrin (1949). β-Ketoadipate was detected by the Rothera reaction (Rothera, 1908).

Chemical syntheses. cis,cis-Muconic acid was synthesized by the method of Elvidge, Linstead, Orkin, Sims, Baer & Pattison (1950b). cis,trans-Muconic acid was prepared from the cis,cis isomer as described by Elvidge et al. (1950a). Racemic muconolactone (λ-carboxymethyl-Δα-butenolide) was synthesized as described by Elvidge et al. (1950b). β-Ketoadipic acid was prepared by the method of Riegel & Lilienfeld (1945). Succinyl-coenzyme A was synthesized by the method of Simon & Shemin (1953).

RESULTS

Enrichment experiments. With different soil samples as inocula, five separate enrichment cultures in media containing cis,cis-muconate as sole source of carbon and energy all yielded one type of non-pigmented aerobic pseudomonad as the dominant organism. The same bacterium was also predominant in four out of five enrichment cultures containing cis,trans-muconate as sole source of carbon and energy. These nine strains were identified by the taxonomic criteria of Stanier et al. (1966), as *Pseudomonas acidovorans.* All strains grew promptly with both cis,cis- and cis,trans-muconate.

Utilization of muconates for growth by Pseudomonas species. The unexpected out-come of these enrichment experiments led us to examine systematically the ability of

Pseudomonas species to grow on muconates. Strains derived from the collection used in the taxonomic analysis of Stanier *et al.* (1966) were employed. No strains representative of the fluorescent groups (*Pseudomonas aeruginosa, P. putida* and *P. fluorescens*) grew immediately with either isomer of muconic acid. When growth did occur, it was delayed, and consisted of a few isolated colonies in an area of dense inoculation on muconate agar plates (Pl. 1, fig. 1). Irrespective of the medium on which they arose, these clones could grow promptly with both *cis,cis-* and *cis,trans-*muconate (Pl. 1, fig. 2). In the light of previous work (Ornston, 1966*a*; Kemp & Hegeman, 1968), they can be interpreted as permeability mutants. The wild types of the fluorescent pseudomonads must accordingly possess the enzymic machinery for the dissimilation of *cis,trans-*muconate, as well as of the *cis,cis* isomer.

Parallel experiments showed that most of the strains of *Pseudomonas acidovorans* and *P. testosteroni* previously studied by Stanier *et al.* (1966) grew promptly and abundantly on both isomers of muconic acid (Pl. 1, fig. 1). Of the 15 strains of *P. acidovorans*, only one (strain 114) failed to grow. Of the 11 strains of *P. testosteroni*, strain 60 could not grow with either isomer, while strains 27 and 78 grew with the *cis,cis*, but not with the *cis,trans* isomer. Since none of these strains had been isolated by methods selective for the ability to dissimilate muconic acids (see Stanier *et al.* 1966), it follows that prompt growth on *cis,cis-* and *cis,trans-*muconic acids is an additional nutritional character that distinguishes the two species of the acidovorans group from fluorescent pseudomads. This nutritional character is not shared by the wild types of the fluorescent pseudomonads, even though they possess the necessary enzymic machinery, since all species of this generic sub-group are impermeable to muconic acids. The situation is analogous to that which exists in the members of the enteric group, with respect to the utilization of citric acid as a carbon source.

Table 1. *Rates of oxygen uptake at the expense of various compounds*
by P. acidovorans strain 13

| | Rate of oxygen uptake (μl./hr/mg. protein) with: | | | | |
Growth substrate	Catechol	Cis,cis-muconate	Cis,trans-muconate	± Mucono-lactone	β-ketoadi-pate
Succinate	0	11	0	0	53
Cis,cis-muconate	248	489	146	232	466
Cis,trans-muconate	149	377	400	208	331

All data corrected for endogenous oxygen uptake.

Muconate metabolism by Pseudomonas acidovorans. One of the newly isolated strains of *P. acidovorans*, obtained by enrichment with *cis,trans-*muconate (strain 13), was used to study the pathway of muconate metabolism by this species. Succinate-grown bacteria did not respire with either isomer of muconate at an appreciable rate. Bacteria grown with either isomer of muconate immediately respired with both isomers at high rates. These rates were equal for bacteria grown with *cis,trans-*muconate, but the respiration with the *cis,trans* isomer by bacteria grown with *cis,cis-*muconate was considerably slower than that of the *cis,cis* isomer. As also shown in Table 1, growth with the muconates likewise induced high rates of oxidation of catechol, racemic muconolactone and β-ketoadipate. These data accordingly suggest that

the muconates can induce all the enzymes required for the metabolism of catechol through the reactions of the β-ketoadipate pathway in *P. acidovorans*.

This conclusion was confirmed by enzyme assays on cell-free extracts, prepared from cultures grown with various substrates (Table 2). Apart from a low level of catechol oxygenase activity, none of the enzymes of the β-ketoadipate pathway was detected in extracts prepared from succinate-grown bacteria. Growth with either isomer of muconate resulted in the induction of muconate lactonizing enzyme, (+)-muconolactone isomerase, β-ketoadipate enol-lactone hydrolase and β-ketoadipate succinyl-CoA transferase. The highest specific activities of these enzymes were always observed in bacteria grown with the *cis,cis* isomer. The specific activity of catechol oxygenase measured in cell-free extracts was low, and the level of this enzyme in muconate-grown bacteria was not significantly greater than in succinate-grown bacteria. However, the manometric data (Table 1) show that a high rate of catechol oxidation by whole organisms is induced as a result of growth with the muconates. The discrepancy suggests that catechol oxygenase was largely inactivated under the conditions of extraction and assay which were used, but this point was not further investigated.

Table 2. *Influence of the substrate used for growth on levels of enzymes of the catechol pathway in extracts from wild type P. acidovorans strain 13*

	Specific activities (units/mg. protein) in extracts from cells grown with:				
Enzyme	Succinate 10 mM	Cis,cis-muconate 10 mM	Cis,trans-muconate 10 mM	β-Ketoadipate 10 mM	p-Hydroxy-benzoate 10 mM
Catechol oxygenase	0·007	0·010	0·010	0·008	—
Cis,cis-muconate lactonizing enzyme	< 0·002	0·132	0·025	< 0·010	—
Muconolactone isomerase	< 0·030	0·530	0·277	< 0·040	< 0·030
β-Ketoadipate enol-lactone hydrolase	< 0·010	0·194	0·125	0·062	< 0·010
β-Ketoadipate succinyl-CoA transferase	< 0·010	0·603	0·152	0·643	—

Both enol-lactone hydrolase and β-ketoadipate succinyl-CoA transferase were induced by growth of *Pseudomonas acidovorans* with β-ketoadipate; there was, however, no significant induction of enzymes specific to the catechol branch of the pathway. Lastly, growth at the expense of *p*-hydroxybenzoate induced neither enol-lactone hydrolase nor muconolactone isomerase. The absence of induction of the former enzyme, essential to the metabolism of *p*-hydroxybenzoate through the β-ketoadipate pathway, is in accord with previous observations (Wheelis *et al.* 1967) that the species of the acidovorans group dissimilate *p*-hydroxybenzoate exclusively through *meta* cleavage of protocatechuate.

The catechol-oxidizing activity of *Pseudomonas acidovorans* involves an oxygenative ring cleavage of the *ortho* type. A crude, cell-free extract of muconate-grown bacteria converted catechol stoichiometrically to a β-ketoacid, identified as β-ketoadipic acid by the characteristic colour given in the Rothera reaction; the same product was produced from *cis,cis*-muconic acid. Highly concentrated crude extracts of bacteria grown with *cis,trans*-muconate, containing 20 to 30 mg. protein/ml., also converted

this isomer of muconic acid to β-ketoadipic acid. The metabolism of the *cis,trans* isomer therefore converges on that of the *cis,cis* isomer.

Properties of mutants of Pseudomonas acidovorans with blocks in the β-ketoadipate pathway. A number of mutants unable to grow with *cis,cis*-muconate were isolated from strain 13 of *P. acidovorans* after mutagenic treatment. Two nutritional classes were partly characterized. One class was unable to grow with either isomer of muconic acid or with β-ketoadipic acid. Analyses of cell-free extracts prepared from cultures grown for four generations in a medium containing 5 mM-succinate, together with 5 mM- *cis,cis*- or *cis,trans*-muconate, showed that such mutants specifically lacked β-ketoadipate succinyl-CoA transferase. The second mutant class had lost the ability to grow with muconates, but could still grow with β-ketoadipate. Analyses of cell-free extracts prepared from cultures grown on succinate and one of the muconates showed that such mutants specifically lacked β-ketoadipate enol-lactone hydrolase. Both these enzymes are, accordingly, essential for the utilization of the muconic acids.

DISCUSSION

Pseudomonas acidovorans can synthesize inducibly the enzymes operative in the catechol branch of the β-ketoadipate pathway, together with enzymes that mediate common terminal step-reactions (specifically, enol-lactone hydrolase and β-ketoadipate succinyl-CoA transferase). The regulation of the synthesis of these enzymes appears similar to that established for fluorescent pseudomonads (Ornston, 1966*b*; Kemp & Hegeman, 1968). Catechol oxygenase is product-induced by *cis,cis*-muconate; both enol-lactone hydrolase and transferase (but not enzymes specific to the catechol branch of the pathway) are induced by growth with β-ketoadipate. With respect both to enzymology and to control, the pathway in *P. acidovorans* resembles the pathway in *P. putida* and *P. aeruginosa*. With respect to physiological function, however, there are notable differences. In fluorescent pseudomonads the catechol branch of the pathway provides a mechanism for the utilization of a variety of aromatic substrates. None of the aromatic substrates utilized by *P. acidovorans* is dissimilated through this sequence of reactions (Wheelis *et al.* 1967). Our observations suggest that in *P. acidovorans* (and probably in the related *P. testosteroni*) the reactions of the β-ketoadipate pathway function physiologically in the dissimilation of two non-aromatic substrates: *viz*, the *cis* isomers of muconic acid. Most strains of both species grow readily with both these compounds, a capacity not found in fluorescent pseudomonads; and *P. acidovorans* can be specifically enriched from soil by the use of either *cis,cis*- or *cis,trans*-muconic acid as a sole source of carbon and energy.

Nevertheless, the induction of catechol oxygenase in *Pseudomonas acidovorans* elicited by the muconic acids shows that this species does possess the potential ability to dissimilate aromatic compounds through the reactions of the β-ketoadipate pathway. The physiological significance of this ability appears questionable, since no metabolic precursors of catechol are known for *P. acidovorans*, and catechol itself is too toxic and too unstable chemically to serve as a normal substrate for growth.

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EXPLANATION OF PLATE

Fig. 1. Growth of Pseudomonas strains of the acidovorans and fluorescent groups on a plate containing *cis,cis*-muconate (0·1 % w/v) as sole source of carbon and energy. The seven strains of *Pseudomonas acidovorans* (24, 29, 61, 62, 102, 129 and 148) grow well on muconic acid. Four of the nine strains belonging to the three species of the fluorescent group do not show visible growth; the remaining five strains yield isolated colonies of permeability mutants. All strain numbers are those used by Stanier *et al.* (1966).

Fig. 2. Growth of *Pseudomonas aeruginosa* strain 277 on 0·1 % (w/v) *cis,cis*-muconate. I. The wild type. II. Permeability mutant isolated on *cis,cis*-muconate. III. Permeability mutant isolated on *cis,trans*-muconate.
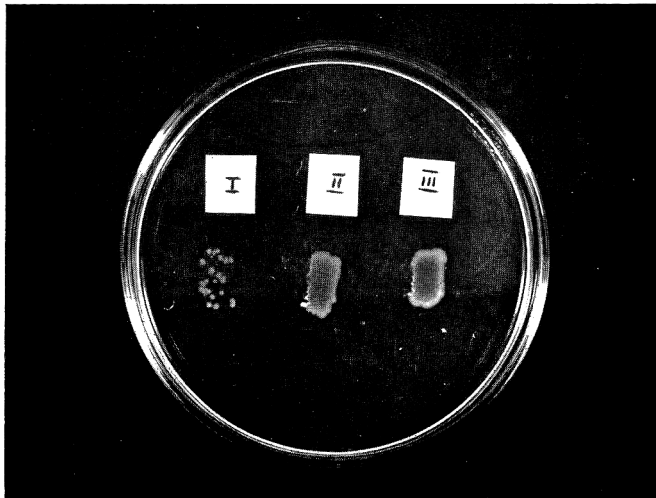
Fig. 1

Fig. 2

The Role of Amine Buffers in EDTA Toxicity and Their Effect on Osmotic Shock

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SUMMARY

The lysis of *Escherichia coli* following exposure to EDTA in tris buffer is similar for most F^+, F^- and Hfr strains. Survival and release of acid soluble nucleotide material was identical in male and female strains. Following osmotic shock by the EDTA + tris method, regrowth patterns of F^-, F^+ and Hfr strains showed no significant differences. Physiological buffers—Tes, Hepes, Bicine—cannot replace tris in EDTA + lysozyme lysis of *E. coli* or in osmotic shock to release surface enzymes. Tes, Hepes and Bicine can replace tris in causing lysis of *Pseudomonas aeruginosa* either with EDTA alone or with lysozyme.

INTRODUCTION

The role of ethylenediaminetetra-acetic acid (EDTA) and amine buffers such as tris (hydroxymethyl) aminomethane (tris) in altering the surface of Gram-negative bacteria and hence permeability has received increasing attention during the last few years. Repaske (1958) demonstrated that some Gram-negative bacteria could be lysed by use of EDTA and lysozyme in a tris buffer. Neu & Heppel (1964) showed that exposure of *Escherichia coli* to tris and EDTA followed by a sudden osmotic shift released a number of degradative enzymes from the bacteria which remained viable. Subsequently, Neu & Chou (1967) showed that an osmotic shock following EDTA + tris treatment resulted in the release of degradative enzymes from most Enterobacteriaceae. It was shown (Leive, 1965) that EDTA caused a non-specific increase in permeability in *E. coli*. Neu, Ashman & Price (1966, 1967) showed that exposure to EDTA could result in breakdown of RNA, and at 4° tris alone caused the nucleotide pool to be lost from various Enterobacteriaceae.

Recently, Goldschmidt, Goldschmidt & Wyss (1967) noted that EDTA and tris toxicity were perhaps related to the fact that male bacteria possess exposed pili. Since we had not noted such striking differences in toxicity between male, female and Hfr strains in our studies on osmotic shock we thought this should be explored. Voss (1967) has shown the role of various compounds in affecting bactericidal activity of EDTA. It seemed of value to determine the role that other physiological hydrogen ion buffers of amine type (Good *et al.* 1966) might play in the role of EDTA toxicity.

METHODS

Organisms. Escherichia coli strains were those previously used in our laboratory and strains that were a generous gift of Dr R. Rudner.

Media and growth conditions. Stock cultures were maintained on Penassay slopes (Difco). Organisms were grown in Penassay broth (Difco) or the high phosphate or low phosphate media of Neu & Chou (1967). Organisms were incubated at 35° on a rapid rotary shaker, and growth was followed by extinction at 600 mμ in a DU spectrophotometer (Beckman). Viability was determined on serial dilutions in Penassay broth, which were plated on either Penassay agar or nutrient agar containing 0·5 % NaCl.

Osmotic shock. Stationary-phase organisms were harvested at 16 hr (1 %, v/v, inoculum). Exponential organisms were harvested at 5×10^8 bacteria/ml.; they were washed twice with room temperature 0·01 M-tris+HCl (pH 7·5)+0·03 M-NaCl or 0·85 % NaCl as noted. Washed organisms were suspended at a ratio of 5×10^9 bacteria/ml. to 80 ml. of 0·5 M-sucrose+0·03 M-tris (pH 7·5). EDTA was added to a concentration of 1 mM for stationary organisms, and after 5 min. of mixing the organisms were removed by centrifugation. The pellet was resuspended at 3° in an equal volume of water for 5 min. The organisms were again removed by centrifugation. Exponential organisms were treated with 0·1 mM-EDTA and resuspended in 0·5 mM-MgCl$_2$ at 3°.

Chemicals. EDTA was used as the disodium salt. Tris was purchased from Sigma and pH adjusted with HCl. Tes, *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid; Hepes, N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid; Bicine, N,N-bis (2-hydroxyethyl) glycine, were purchased from Calbiochem.

Enzyme assays. 5'-Nucleotidase, cyclic phosphodiesterase and inorganic pyrophosphatase assays are those previously published (Neu, 1967).

RESULTS

Previous studies of osmotic shock of Enterobacteriaceae had not shown differences in F$^+$ and F$^-$ strains in our laboratory (Neu & Chou, 1967). Table 1 shows that when overnight cultures of various *Escherichia coli* strains were exposed to 1 mM-EDTA at

Table. 1. *Effect of buffers on lysis of various male and female Escherichia coli strains*

	Buffer		
Strain	Tris	Hepes	Tes
		Per cent A$_{600}$ change	
Hfr Hayes	4·9	1·4	0
CR 34 (F$^-$)	3·2	3·1	2·2
CR 161 (F$^+$)	18	0	0
W 1192 (F$^+$)	16	13	26
Hfr 3000	7	+4	+3
W 1485 (F$^-$)	14	0	0
W 1117 (F$^-$)	5	3	—

Overnight cultures were washed with 0·03 M-NaCl +0·01-M-tris-HCl and resuspended at 21° in 0·03 M-buffer at 10^{10} bacteria/ml. Change in extinction was followed for 15 min. at 600 mμ.

21° there seemed to be increased lysis of some F$^+$ strains as compared with some F$^-$ strains. Hfr strains seemed as stable as the F$^-$ strains, to the effect of EDTA in tris buffer. Two other buffers, Hepes and Tes, showed much less lysis than did tris.

A comparison of the survival of eight strains of *Escherichia coli* after osmotic shock was made. No differences were noted in release of acid soluble material, release

Table 2. *Effect of buffer on EDTA + lysozyme lysis of Escherichia coli*

Buffer (pH)	Change in extinction CR34 (F⁻)		Survival	Nucleo-tidase release	Change in extinction C4 (F⁻)		Survival	Nucleo-tidase release	Change in extinction CR161 (F⁺)		Survival	Nucleo-tidase release
	(%)	A_{280}	(%)	(%)	(%)	A_{280}	(%)	(%)	(%)	A_{280}	(%)	(%)
Tris (7·4)	93	0·329	2	100	90	0·309	10	98	73	0·374	22	100
Hepes (7·1)	19	0·137	100	28	5	0·245	73	20	19	0·161	78	32
Tes (7·6)	50	0·184	85	66	10	0·206	98	34	28	0·240	75	48
Bicine (7·4)	31	0·148	73	42	13	0·319	71	50	18	0·192	60	29

Organisms were grown to early stationary phase in tris medium. They were washed twice with 0·85% NaCl at 21°. 10^{10} bacteria were suspended in 0·03 M-buffer. EDTA concentration was 1 mM and lysozyme 10 μg./ml. Change in extinction was at 600 mμ. A_{280} release was determined on the supernatant fluid of 10 min. treated bacteria.

of surface enzymes or survival of WI485 (−), CRI6I (+), KIO (−), WIII7 (−), AB1192 (+), AB282 (+), P4×6 (Hfr) and Hfr. Hayes. Growth of bacteria in either the high phosphate or low phosphate media did not alter the results. Growth patterns after osmotic shock of F−, F+ and Hfr strains were identical.

Comparison of male and female strains in relation to time of exposure and type of buffer demonstrated that F+ and Hfr strains had a decreased survival after exposure to 0·03 M-Tes+0.25 mM-EDTA for 60 min. Tris and Bicine buffers did not show increased toxicity as time of exposure to EDTA and the buffer was increased.

Table 3. *Effect of buffer upon release of enzymes from Escherichia coli by osmotic shock*

	Per cent released					
Buffer (pH)	5′-Nucleotidase	Acid phosphatase	3′-Nucleotidase	Inorganic pyrophosphatase	A_{260}	Survival (%)
Tris (7·4)	90	58	75	2	0·435	84
Tes (7·6)	20	30	34	3	0·435	92
Hepes (7·1)	10	25	33	7	0·423	100
Bicine (7·4)	21	26	38	10	0·448	97

An overnight culture of K37 was subjected to osmotic shock as outlined in Methods. Inorganic pyrophosphatase is used as an internal enzyme to detect cell damage.

Table 4. *Effect of buffer on EDTA toxicity for Pseudomonas aeruginosa*

	Change in extinction (%)		A_{260} EDTA	Survival (%)	
Buffer	EDTA	EDTA+Lysozyme		EDTA	EDTA+Lysozyme
Tris	25	81	0·796	34	10
Tes	18	24	0·715	62	9
Bicine	15	24	0·923	46	12

An early stationary-phase culture was harvested at 21° and washed twice with 0·85% NaCl. Washed organisms at a concentration of 10^{10} bacteria/ml. were exposed to 0·1 M-EDTA in the buffers at 0·03 M at a pH of 7·5 at 21°. Change in extinction was read at 10 min. at 600 mμ. A_{260} release was determined on supernatant of treated cells. Bacteria treated with lysozyme at 20 μg/ml.

The effect of various buffers on the EDTA+lysozyme lysis of *Escherichia coli* (Table 2) show that lysis was significantly less with all the other physiological buffers at a similar pH for both male and female *E. coli*. Survival correlated with the lower release of acid soluble material and release of surface enzymes as represented by 5′-nucleotidase. An identical response was seen in the comparison of the same buffers used in osmotic shock to release surface enzymes. The other buffers—Tes, Hepes, Bicine—did not adequately replace tris (Table 3).

Washing of bacteria with either 0·85 % NaCl or 0·01 tris-HCl (pH 7.4) did not affect the EDTA+lysozyme lysis with tris as the buffer. Similarly, bacteria washed with tris and then suspended in Tes, Bicine or Hepes and EDTA did not show more lysis than NaCl-washed bacteria.

Treatment of Aerobacter showed that Hepes and Bicine were as ineffective as tris in promoting EDTA+lysozyme lysis of Aerobacter strains. Aerobacter did however release surface enzymes with osmotic shock (Neu & Chou, 1967).

Although Tes and Bicine were not effective in replacing tris in EDTA lysis of *Escherichia coli*, they were as effective in causing lysis of *Pseudomonas aeruginosa* either with EDTA alone or with lysozyme (Table 4).

DISCUSSION

Although the results of Goldschmidt *et al.* (1697) suggested that all male strains of *Escherichia coli* were more sensitive to the toxic effects of EDTA + tris, we have been unable to confirm this. Some strains did show increased toxicity as compared with most female strains, but in general toxicity was similar for F$^+$, F$^-$ and Hfr strains. Occasional male strains that did show increased toxicity may have had defects of transport as indicated by their delayed regrowth following osmotic shock.

It is clear that tris has an effect on the bacterial cell wall unlike similar buffers such as Tes, Bes, Bicine and Hepes. Although these buffers can replace tris in other systems, they cannot be used to cause EDTA + lysozyme lysis or in the osmotic shock treatment to cause release of surface enzymes and transport factors from Enterobacteriaceae.

Gray & Wilkinson (1965) have shown that *Pseudomonas aeruginosa* is sensitive to EDTA in a variety of buffers. All of the buffers used—Tes, Hepes, Bicine—were effective in causing EDTA lysis.

These studies illustrate that tris cannot be replaced by other buffers in the systems using EDTA to alter permeability of bacteria except in very sensitive organisms. It is possible either that these other buffers form weaker homologues with EDTA than tris or that they have less ready access to the cations which stabilize the cell. The mechanisms of the different responses to buffers other than EDTA in *Escherichia coli* are under study.

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Ultrastructure of an Anaerobic Filamentous Oral Micro-organism

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SUMMARY

Thin sections of an anaerobic filamentous oral organism possibly related to *Leptotrichia buccalis* have been examined in the electron microscope. The organism exhibited a cell wall profile characteristic of Gram-negative bacteria, *viz.* a solid membrane and an outer double-layered membrane. The outer membrane could be removed by treating intact cells with trypsin or phenol-water. The organism contained numerous intracytoplasmic vacuoles.

INTRODUCTION

Morphological and physiological properties of an anaerobic filamentous micro-organism isolated from the human oral cavity have been reported (Hofstad, 1967*a*). This micro-organism, which proved to be identical to a filamentous bacterium de-scribed by Theilade & Gilmour (1961), appeared to be related to *Leptotrichia buccalis* (Gilmour, Howell & Bibby, 1961), but differed from that species serologically and in showing a heterofermentative type of sugar metabolism. Like *L. buccalis*, very young cells of the atypical organism were Gram-positive, while most cells in cultures in the stationary phase of growth were Gram-negative.

In the electron microscope, *Leptotrichia buccalis* exhibits a cell wall profile charac-teristic of Gram-negative bacteria (Hofstad & Selvig, 1969). The fine structure of the related atypical filamentous micro-organism has been reported by Theilade, Theilade & Scott (1962). These authors did not, however, study the cell wall structure of this organism.

The present report concerns the ultrastructure of the filamentous micro-organism as revealed by electron microscopy of intact cells and cells treated with trypsin or phenol-water, with particular attention to the cell wall profile.

METHODS

The isolation of the filamentous organism, strains L44 and L49, has been described earlier (Hofstad 1967*a*). Cultivation was performed in enriched nutrient broth (Hofstad 1967*a*), and the bacteria were harvested by centrifugation when in exponen-tial phase of growth (1 or 2 days at 37°).

In preliminary experiments the harvested bacteria were washed with saline or phosphate buffer at neutral pH. However, the washings caused many organisms to break, and the remainder appeared fuzzy and with poorly resolved cell wall layers

when examined in the electron microscope. Therefore, the washing was omitted and subsequent preparations were carried directly to fixative.

Trypsin digestion was done on washed bacteria with crystalline trypsin (Trypure Novo, Novo Industri A/S, Copenhagen), freshly prepared in 0·02 M-tris buffer of pH 7·8, using 10 mg. trypsin/g. packed wet bacteria. The digestion was performed at 37° for 20 hr. The digested bacteria were centrifuged and washed twice in saline.

Treatment with phenol-water was carried out by homogenizing equal volumes of an aqueous suspension of 200 mg. wet packed bacteria and 90 % phenol for 15 min. at 4°.

Following centrifugation the water and phenol phases were pipetted off and the treated bacteria washed once in distilled water.

Pellets of intact or treated bacteria were resuspended in acetate + veronal buffered osmium tetroxide fixative, transferred to agar and embedded in polyester resin according to the procedure suggested by Kellenberger, Ryter & Séchaud (1958). Thin sections for electron microscopy were cut with glass knives, collected on carbon-coated formvar membranes, and stained with 2 % aqueous phosphotungstic acid for 30 min., or with lead citrate (Reynolds, 1963) for 5 to 15 min. Some bacteria were resuspended in distilled water. Drops of the suspension were placed on specimen support grids coated with carbon and formvar membranes, dried and shadowed with palladium from an angle of 20 degrees. Specimens were examined in the electron microscope and photographed at plate magnifications of 5,000 to 20,000 diameters.

RESULTS

Intact organisms

The anaerobic filamentous organism, strains L44 and L49, appeared in thin sections as elongated structures whose shorter diameter ranged from 0·4 to 0·6 μ (Pl. 1, fig. 1). The bacteria had rounded ends and were often adhering end to end. A moderate amount of floccular substance was seen adhering to the surface and in the surrounding medium.

The concentric membranous structures surrounding the cytoplasm will be referred to as the plasma membrane, solid membrane and the outer membrane. The plasma membrane appeared as a double structure, 70 to 80 Å in width, which followed a straight or slightly wavy course (Pl. 1, fig. 2).

The solid membrane appeared as a distinct single electron-dense line, less than 50 Å in width (Pl. 1, fig. 2). It followed the course of the plasma membrane, separated from it by a space of 50 to 150 Å. This space contained varying amounts of granular electron-dense material.

The outer membrane was separated from the solid membrane by a less electron-dense layer, around 40 Å in width. Where the multi-layered cell wall was best resolved, the outer membrane could be seen as a double-tracked structure. In other organisms the outer membrane appeared as a less distinct single line. The outer membrane was regularly coated by a more or less dense crust of granular or floccular material (Pl. 1, fig. 2).

The cytoplasm consisted mainly of an evenly dispersed electron-dense granular material. Nuclear regions appeared as less electron-dense regions filled with fibrillar substance (Pl. 1, fig. 1). Many cells contained oval or circular vacuoles (Pl. 1, fig. 1). The contents of the vacuoles were less electron-dense than the surrounding cytoplasm.

The filamentous shape of the micro-organism was confirmed in shadowed preparations. Some of the organisms were collapsed; these showed numerous rigid bodies which were interpreted as corresponding to the intracytoplasmic vacuoles (Pl. 1, fig. 3).

Organisms treated with trypsin or phenol-water

Following digestion with trypsin, most bacteria maintained their original shape, but the wall structure was considerably modified (Pl. 1, fig. 4). Generally, the cell wall consisted of a single electron-dense layer separated from the plasma membrane by a space of varying width. This space did not contain appreciable amounts of stainable material.

After treatment with phenol-water the organisms were extensively altered (Pl. 1, fig. 5). The cell content appeared as a homogeneous mass retracted from the wall. The cell wall appeared as a single line, frequently with attached outer cell wall material.

DISCUSSION

The cell wall profile of the filamentous oral organism conforms to the general description of the Gram-negative cell wall type (Murray, Steed & Elson, 1965; de Petris, 1965), and is characteristically different from the cell wall profile of Gram-positive bacteria (Glauert, 1962; Murray, 1962). The various layers were not as clearly differentiated, however, as in the possibly related species *Leptotrichia buccalis* (Hofstad & Selvig, 1969).

The finding of a Gram-negative cell wall type is in agreement with previous chemical studies of isolated walls (Hofstad, 1967*b*). Cell walls of the filamentous organism contained a wide range of amino acids, and protein and lipid accounted for approximately 50 and 20 % of the dry weight, respectively. In contrast, the hexosamine content did not exceed 5 %. Furthermore, the cell wall preparations contained an aldoheptose, a component previously demonstrated in Gram-negative bacteria only.

The disappearance of the outer membrane from the trypsin-treated organisms indicates that this membrane of the filamentous organism is made up mainly of protein. This hypothesis is supported by chemical analysis of the material extracted from washed whole bacteria or cell wall preparations by digestion with trypsin. The extracted material contained 55 % by weight protein, 25 % lipid and 20 % neutral sugars (T. Hofstad, unpublished results).

The removal of the outer membrane by trypsin digestion is somewhat at variance with the results of de Petris (1967), who reported on the cell wall structure of *Escherichia coli*. Digestion of intact or heat-treated organisms with proteolytic enzymes caused no removal of the outer membrane in this species. Rather, the double-layered outer membrane was sharply outlined and apparently unaffected by the treatment. On the other hand, this membrane could be removed by treatment with phenol-water, which in the present study was less effective than trypsin treatment in this respect.

Trypsin-digested cell walls of the filamentous organism contain approximately 25 % amino acids, 38 % neutral sugars and 4 % fatty acid esters (Hofstad, 1967*b*). This indicates that the solid membrane comprises structures other than pure mucopeptide.

The removal of the stainable material between the plasma membrane and the solid membrane by digestion with trypsin shows that protein is interposed between these

membranes. Analogous findings have been made in *Escherichia coli* (Weidel, Frank & Martin, 1960; de Petris, 1967).

The nature of the intracytoplasmic vacuoles is not clear. However, similar structures, interpreted as intracellular polysaccharide, have been found in cariogenic streptococci (Guggenheim & Schroeder, 1967) and in coccoidal, rodshaped and filamentous bacteria in human dental plaques overlying various and, to a lesser degree, normal enamel (Frank & Brendel, 1966). The finding of rigid bodies within collapsed bacteria supports the assumption of a consolidated content of these vacuoles.

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EXPLANATION OF PLATE

Fig. 1. Thin section of the anaerobic filamentous oral organism (strain L44). The cytoplasm is surrounded by three concentric structures: the double-tracked plasma membrane, the solid membrane and the double-tracked outer membrane. Numerous intracytoplasmic vacuoles are present, especially in the bacterium to the right. Small amounts of floccular material adhere to the cell surface. N—nuclear regions. Magnification × 40,000.

Fig. 2. Detail from Fig. 1. PM—plasma membrane, SM—solid membrane, OM—outer membrane. × 100,000.
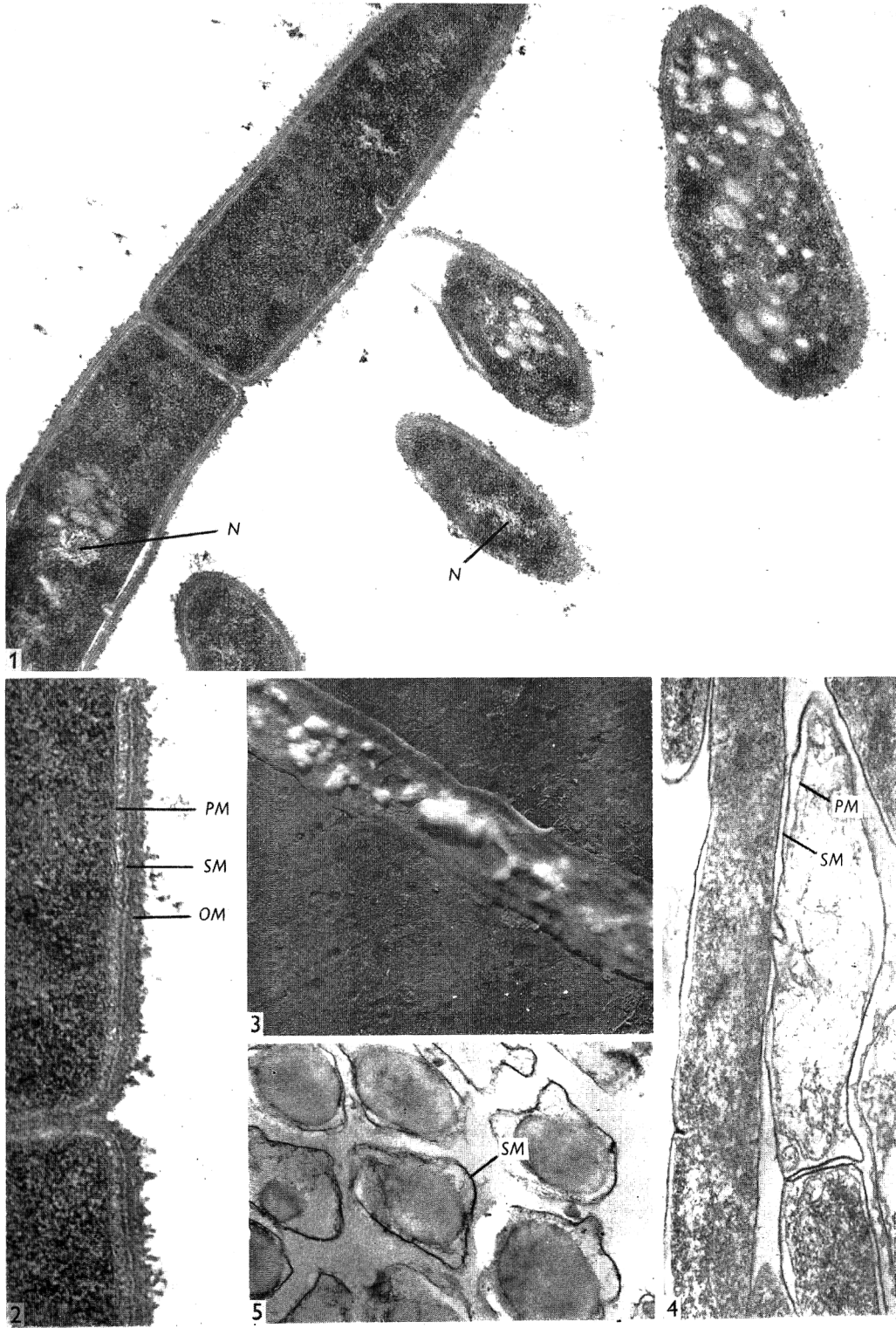
Fig. 3. Shadowed specimen (strain L49). The collapsed bacterium contains intracytoplasmic rigid structures. × 10,000.

Fig. 4. Result of trypsin digestion (strain L44). The bacteria have maintained their shape, but the outer membrane is missing. PM—plasma membrane, SM—solid membrane. × 40,000.

Fig. 5. Result of treatment with phenol-water (strain L44). The cell wall appears in general as a single electron dense line which corresponds to the solid membrane (SM). × 40,000.

Thioguanine-dependent Light Sensitivity of Perithecial Initiation in *Sordaria fimicola*

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SUMMARY

Perithecial initiation is strongly inhibited by visible light in less than 18-hr-old hyphae of *Sordaria fimicola* which are grown in the presence of 1 μM-6-thioguanine. It is known that 6-thioguanine is destroyed when exposed to light *in vitro* with oxygen and a sensitizing dye like methylene blue is present. The most effective wavelength region for perithecial inhibition is the blue region, indicating that possibly flavins are the photosensitizing compounds in this case. The synthesis of carotenoid and melanin pigments is also affected by blue light in this organism. Another, transient, inhibitory effect on perithecial production is found when light-grown cultures are transferred to darkness. As a consequence of the two light effects, mycelia exposed to alternating light and dark periods in growth tubes develop well-defined zones of perithecia. A simple state-input model is presented in the Appendix to demonstrate how such zonation patterns can be generated on the basis of the light effects and a few other assumptions.

INTRODUCTION

Sordaria fimicola is a homothallic ascomycete in which the development of its sole reproductive structure, the perithecium, is not sensitive to light (Hawker, 1957). Alternating light and darkness affect the spore discharge from mature perithecia of this organism (Ingold & Dring, 1957; Walkey & Harvey, 1967) but not their initiation, although the perithecia may attain a larger size under alternating conditions than under continuous light or continuous darkness. In the course of previous work (Lindenmayer & Schoen, 1967) it was found that certain purine and pyrimidine analogues, and particularly 5-fluorouracil, could specifically inhibit perithecial initiation in this organism at concentrations at which only slight inhibition of vegetative growth was observed. We also noticed a marked sensitivity of perithecial production to light-dark conditions when the organism was grown in the presence of 1 to 10 μM-6-thioguanine or 8-azaguanine. None of the other nucleic acid base analogues produced a similar photodynamic effect. It is known (cf. Shapiro, 1968) that guanine and many of its derivatives are decomposed by visible light *in vitro* in the presence of molecular oxygen and of catalytic amounts of certain dyes, like methylene blue. It has also been reported (Friedman, 1968) that 6-thioguanine is one of the compounds so affected, but apparently 8-azaguanine is not. The main absorption band of 6-thioguanine at 323 mμ (it has no bands in the visible region) disappears as a result of a few minutes of illumination of rather low intensities in the presence of O_2 and methylene blue, and no

other bands appear, indicating the destruction of the purine ring (Friedman, 1968). Since our experiments showed an *in vivo* photodynamic effect of these compounds, involving presumably a native cellular pigment as the photosensitizing dye, and since the effect consists of the specific suppression of an important morphogenetic process, we decided to investigate this system. But only 6-thioguanine was used in our experiments because 8-azaguanine gave rise to irregular vegetative growth, while 6-thioguanine did not inhibit vegetative growth at all, in fact it stimulated growth somewhat (Lindenmayer & Schoen, 1967).

METHODS

A wild-type strain of *Sordaria fimicola* (Rob.) Ces. et de Not. was obtained from the American Type Culture Collection (strain 14517).

The culture techniques and the medium, aside from the addition of 1 μM-6-thioguanine before autoclaving, were the same as in the previous work (Lindenmayer & Schoen, 1967). The medium was a sucrose nitrate medium, containing biotin and thiamine (pH 5·5 to 6·0), and is known to stimulate the production of perithecia (Bretzloff, 1954). To obtain linear surface growth of the fungus, Pyrex screw-cap test tubes (15 cm. long, 1·2 cm. diam.) were half-filled with the molten agar medium, which was then allowed to solidify while the tubes were in a horizontal position. The average linear growth rate of the fungus was 1·5 cm. per day, thus there was enough room for 10 days of growth in each tube. All of the experiments were made in a plant growth chamber (Sherer-Gillet model CEL 34-7HT) at constant temperature and illumination. The temperature was kept at 24°. White light was supplied by four Sylvania F48 T12-110 V/CW VHO fluorescent tubes and four 50 W incandescent bulbs. The culture tubes were placed horizontally at a level 30 cm. below the light sources, and were separated from them by the heavy glass plate enclosing the chamber. The white light intensity at the level of the cultures was estimated as 600 to 800 footcandles. Illumination by light of particular wavelength regions was achieved by using 'Cinemoid' colour filters of known transmission characteristics (supplied by Kliegl Bros., Long Island City, N.Y.). No attempt was made to compensate for the reduction of light intensity under the filters, since even the transmitted intensity was high enough for the effects tested. Cultures were given dark treatment by placing them in light-tight containers in the same growth chamber.

The figures are shown in half natural size with respect to the lengths of the tubes, but their diameters are somewhat further reduced. The dots represent individual perithecia as they were observed at the end of experiment, i.e. at the time when the last boundary line was drawn. Each day at the same hour the advancing front of the colony was marked on the tube, and these boundaries are shown in most figures. Growth took place from left to right. The L and D symbols designate light or darkness to which the advancing mycelium was first exposed in a given interval. It is important to realise that the whole length of each tube was always exposed to light or darkness, thus any given section may have been treated by several cycles of light and dark, and the L or D symbol refers only to the first treatment that section received while the mycelial front was moving through it. Each treatment was administered to pairs or triplets of growth tubes, and each experiment was repeated at least once. The figures show representative samples.

RESULTS

First we report on experiments with thioguanine-free cultures, in order to establish the normal behaviour of this organism under alternating light-dark treatments. The results are shown in Fig. 1. It is seen (tubes A and B) that perithecia were formed under continuous white light, as well as under continuous darkness (6 days each), although somewhat fewer under light. When cultures were grown in light for 4 days and then transferred to darkness (tube C), there was an inhibition of perithecial initiation from
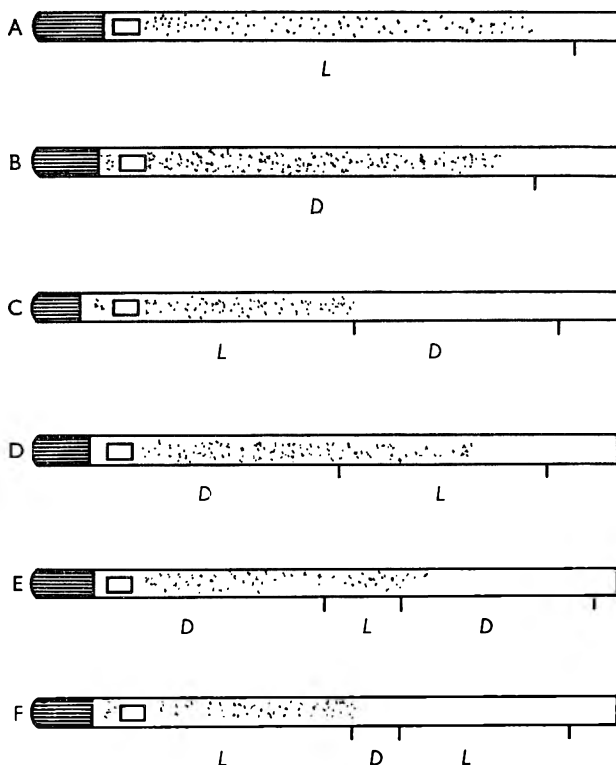
Fig. 1. Effects of white light or darkness on thioguanine-free cultures of *Sordaria fimicola* with respect to production of perithecia. The light or dark treatments were administered to the entire lengths of the growth tubes, beginning or ending when the advancing front of the mycelium was at the vertical lines shown. Inoculum at left. Tubes E and F received 24 hr of light or darkness, respectively, after 4 days of growth.

the boundary line on. This inhibition was only temporary, however, as evident from other experiments, and extended usually for not longer than 1 day's growth (in the figure microscopic initials are not shown). When 4 days old dark-grown cultures were transferred to light (tube D), there was no inhibition at the boundary line, in contrast to the findings with thioguanine-grown mycelia. Administering a 1-day light period to dark-grown cultures (tube E) gave rise to an inhibition to perithecial formation in the second dark-grown section. One day of darkness given to light-grown cultures (tube F) also showed an inhibition beginning with the start of the dark period. The transient inhibition of perithecial initiation upon passage from light to dark on regular growth medium (and also on thioguanine-containing medium, as is shown below) is a pheno-

menon not previously reported, which was, however, not investigated any further in this study.

The initial experiment with 6-thioguanine is shown in Fig. 2. In this experiment alternating 24 hr periods of light and darkness were administered to the growth tubes, which in this case were 30 cm. long. The perithecial zonation which developed as a result is shown as it appeared at the end of the experiment (the time of the last boundary position). Each zone was formed in the later part of a light-grown interval, with a rather sharp cut-off at the boundary with the following dark-grown portion. The light-grown sections were noticeably pink coloured, while the dark-grown ones were colourless at first, with fluffy aerial hyphae present on them.

After the completion of growth, the whole agar content of the tube was allowed to
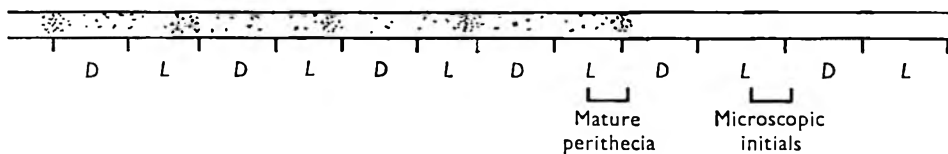

Fig. 2. Perithecial zonation in a culture containing 1 μM-6-thioguanine under alternating periods of 24 hr light and 24 hr darkness. The regions where mature perithecia and perithecial initials are indicated were determined by microscopic observations.

slide out (after flaming the entire length of the tube for a short time), and the mycelium of the last 4 days of growth was examined microscopically for perithecial initials. The very young perithecial initials are hook- or coil-shaped structures, as described by Greis (1936). As indicated in Fig. 2, the youngest initials could be seen in the second half of the last but one light-grown section.

From this experiment alone it could be concluded that in thioguanine-grown cultures light is necessary, or darkness is inhibitory, for perithecial initiation. Thioguanine-free cultures grown under an alternating 24 hr light-dark regime do not produce zones. Further experiments demonstrated that far from being stimulatory light was strongly inhibitory for perithecial production in hyphae less than 18 hr old. The reason for the zones arising in the light-grown sections is the 18 hr lag between the initiation of a hypha and that of a perithecium borne by it.

That this is so is demonstrated by the next set of experimental tubes, which were given the same light-dark treatments as those in Fig. 1, but contained 1 μM-6-thioguanine. Under continuous light (Fig. 3, tube A), perithecia were almost completely absent, in contrast to the case of the controls (Fig. 1, tube A). In continuous darkness (tube B) the number of perithecia formed was, on the other hand, similar to that of the control. When light-grown cultures were switched to the dark (tube C), perithecia appeared on the originally light-grown portion, first near the boundary, then extending more sparsely further back. Following the light-dark boundary there was a short inhibited zone, with normal perithecial density subsequent to that. When dark-grown cultures were switched to light (tube D), perithecial production was inhibited backwards from the boundary line for a distance of about 18 hr growth, and it remained inhibited for the length of the light-grown portion. Administering 24 hr light exposure to dark-grown cultures (tube E) resulted in a similar inhibition zone extending back from the boundary line, and also forward from it through half of the light-grown section. Some inhibition was also apparent after the second boundary line, in the

beginning of the second dark period. Finally, in the reciprocal experiment (tube F), a perithecial zone appears before the first boundary line, on the last section of the light-grown portion. This zone corresponds very closely in its appearance to those in Fig. 2.

It is clear that in thioguanine-grown mycelia perithecial initiation was inhibited by light in hyphae exposed for the first 18 hr period of their life. On the other hand, if hyphae which were produced in the light were switched to the dark before they were
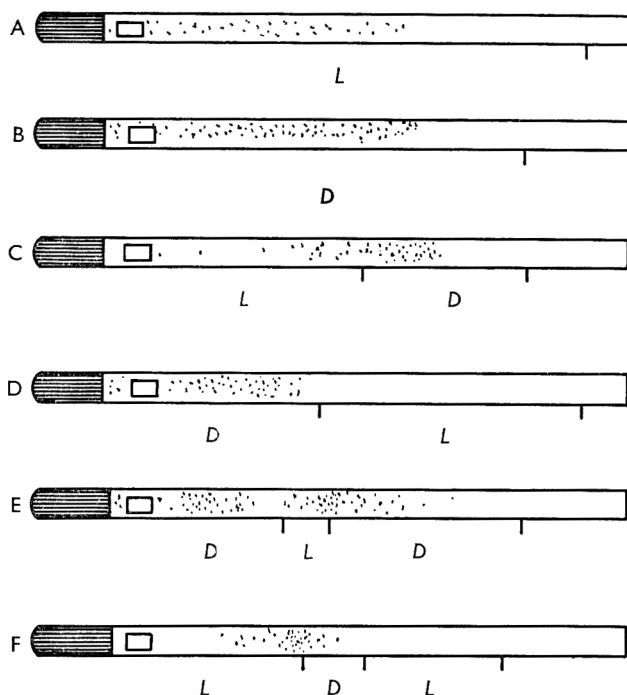
Fig. 3. Effects of white light or darkness on perithecial initiation in cultures containing 1 μM-6-thioguanine. Same light and dark regimes as in Fig. 1.

18 hr old, they still produced perithecia. The microscopic observations in Fig. 2 confirm this: the last light-grown section cannot have perithecia because it was too young, the preceding dark-grown section cannot have any because most of it was exposed to light when it was still less than 18 hr old, so the first place where perithecia could arise was in the last-but-one light-grown section which received darkness while it was photosensitive. To show that this reasoning can explain the zonation found under alternating 24 hr light-dark regime see computation in Appendix. Another conclusion is that the transient inhibition following the switch from light to dark is observable in thioguanine-containing cultures, although the extent of this inhibition seems to be smaller than in the thioguanine-less cultures.

In order to establish the minimum length of light exposure necessary for the inhibition of perithecia in the presence of thioguanine, various periods of light of constant intensity were administered to dark-grown cultures. These experiments indicated that 8 hr of exposure produced a zone of inhibition as extensive as the one produced by

24 hr exposure. Shorter light periods, down to 1 hr, gave rise to shorter but still well-defined zones of inhibition immediately preceding the boundary. Exposures for 10 and 30 min. did not produce any inhibition.

Experiments were performed to find out which wavelength region was most effective in inhibiting perithecia. Thioguanine-containing cultures were exposed to light, either continuously for several days or for a 24 hr period between longer dark periods, under a series of 'Cinemoid' colour filters. The following filters were used: blue—No. 32 (transmission up to 560 mμ and some around 700 mμ), green—No. 39 (transmission between 460 and 580 mμ), yellow—No. 1 (transmission above 460 mμ), orange—No. 5A (transmission above 525 mμ) and a combination of red and yellow filters—No. 6 and 1 (combined transmission above 580 mμ, the red filter alone also transmits in the blue region). Blue light was found to be most effective, and, although the total transmission of the blue filter is only 7 % of the incident light, the perithecial inhibition was the same under this filter as under direct white light. The yellow and orange filters gave a certain amount of inhibition but much less than the blue filter. The green and red filters showed the least effect. Thus the blue region of visible light seemed definitely the most effective, and the red region the least. The fact that more inhibition was observed under yellow or orange filters than under green may perhaps be explained by the much lower total transmission of the green filter (12 %) than of the yellow (90 %) or the orange filter (39 %). But it is also possible that the photosensitive pigment has two absorption maxima, one in the blue and the other in the yellow region of the spectrum. It should be pointed out here that ultraviolet light could not have been responsible for producing the inhibitory effect, since the cultures were separated from the fluorescent source by a heavy glass plate. It is also clear that the absorbing pigment could not be 6-thioguanine itself, since it does not absorb in the visible region.

Blue light is also responsible for the synthesis of carotenoids in this organism. Their presence was first reported in Sordaria by Ingold & Hadland (1959), and we could confirm this by obtaining typical carotenoid absorption spectra in intact mycelia with a Cary 14 spectrophotometer (Surapipith, 1969). The mycelia grown under white light or under blue or green filters had very large absorption bands at 430, 460, 490 and 520 mμ, while those grown in the dark, or under yellow, orange or red + yellow filters had only slight peaks in that area, and exhibited absorption bands in addition at 420 mμ (the main Soret band of the cytochromes which was masked in the spectra of carotenoid containing cells) and at 320 and 360 mμ in the ultraviolet (presumably due to colourless carotene precursors like phytofluene; cf. Goodwin, 1965). The necessity for blue light for carotenoid production in this organism closely resembles the situation in *Neurospora crassa*, as it was demonstrated by Zalokar (1955).

Another pigment, a green-black melanin-type pigment, is also affected by light in *Sordaria fimicola*. This pigment is only formed in the dark in the vegetative mycelium (it is presumably the same pigment which makes the perithecia and ascospores black, where it is produced in the light as well). Even 10 min. exposure to white light of thioguanine-grown cultures produced a pigment-less zone which extended back to the mycelium which developed in the two previous days. Thus the light requirement for inhibiting the synthesis of this pigment was much lower than that for inhibiting perithecial production. Blue light was most effective in this case also. Whether there is any connexion between the shutting off of carotenoid production and the start of melanin production in the absence of blue light, is an interesting problem. The black pigment

of the vegetative mycelium as well as of the perithecia turns yellow when placed in sodium dithionite solution, a strong reducing agent, and turns black again when reoxidized by H_2O_2, indicating the presence of quinone groups and conforming to the reactions of allomelanins as described by Nicolaus (1968).

DISCUSSION

The following assumptions seem to be sufficient to account for the zone patterns obtained with the various light-dark treatments of cultures grown on medium containing 6-thioguanine.

(1) In darkness the newly formed hyphae undergo progressive transformations until they are 18 hr old, when they become determined for bearing perithecia. The first stages of initials are, in fact, observable microscopically on hyphae of about this age, and not on any younger ones.

(2) Under blue light of sufficient intensity the newly formed hyphae undergo progressive changes as well, but these lead after 18 hr to a state in which perithecia cannot be induced even by subsequent dark periods.

(3) If a culture is switched from light to darkness, the hyphal segments which are at that time in intermediate light-grown states change to the age-wise corresponding intermediate dark-grown states, except for short segments at the hyphal tips which become unable to form perithecia. Thus, perithecia will eventually appear as far back as 18 hr growth from the boundary line, but there will be a narrow zone of inhibition immediately after the line.

(4) If a culture is moved from darkness to light, the hyphae in intermediate light-grown states are transformed into intermediate dark-grown states, but in reverse age order. In other words, the youngest dark-grown state is transformed into the most advanced light-grown state, and the most advanced dark-grown state goes to the least advanced light-grown state. The reverse order needs to be assumed in order to account for the fact that shorter than 18 hr light periods induce proportionately shorter zones of inhibition, that these zones develop immediately next to the boundary line, and that the maturation of perithecia spreads backwards from the line marking the start of the dark treatment.

In addition to perithecial zonation under single light-dark transfers, or under single light or dark exposures, these assumptions can also account for the zonation pattern found under 24 hr light-dark alternating regime, as well as for zonation under regimes of shorter alternations (for a demonstration that these assumptions are sufficient see the Appendix below; it does not follow, however, that they are necessary). With respect to the findings in Fig. 2 we have already discussed the appearance of perithecial bands at the ends of light-grown sections with sharp cut-offs towards the boundaries with the dark-grown portions, obtained under 24 hr alternating light and dark periods. Preliminary experiments were also done on 12 hr alternations, in which case a narrow perithecial band develops after each dark/light boundary, one every 24 hr. Similar experiments with 6 hr alternating periods resulted in no bands of perithecia.

The assumptions listed above are reducible to two light-effects on perithecial initiation: the thioguanine-induced inhibitory effect of blue light, and the transient inhibition of perithecia following a light-to-dark transfer. The molecular mechanism of the first effect poses interesting questions. First of all, by analogy to the *in vitro*

destruction of thioguanine in the presence of photosensitizing dyes (Friedman, 1968), a blue-absorbing compound must be present in the cells having a similar function. The two main classes of compounds with absorption in the blue region are the carotenoids and the flavins. The carotenoids seem to be ruled out, since perithecial inhibition can be observed when dark-grown cultures are exposed to 1 hr of light, and since the formation of carotenoids itself must be induced by light, therefore, there does not seem to be sufficient time for the newly produced pigments to act on thioguanine. This leaves the flavins and flavoproteins as the most likely photosensitizing pigments, and their role is planned to be further investigated. Flavoproteins are the most likely candidates for many of the blue-sensitive reactions in fungi, including the induction of carotenoid synthesis (Zalokar, 1955; Rau, 1967).

The other question concerning the thioguanine effect is how the photo-destruction of thioguanine results in the inhibition of perithecial production but not of growth. Thioguanine may be decomposed either before it gets incorporated into nucleic acids, or after that, but in both cases the resulting changes should be equally deleterious to vegetative metabolic processes as to reproductive ones. A possible explanation may be that a certain kind of guanine-containing compound, which is essential for the initiation of perithecia, is more likely to come in contact with the photo-sensitizing pigment than the other nucleic acids. But it cannot be simply assumed that this compound essential for perithecia is a newly synthesized messenger RNA, since then all other newly synthesized messenger RNAs which are necessary for continued vegetative growth would also be inactivated. The compound involved in perithecial initiation must somehow be more exposed to photo-inactivation than the other guanine-containing cell components.

APPENDIX

A. Lindenmayer

The simple automata theoretical model which is presented here employs the principles for systems with state transitions and input-output relationships which are discussed in detail by Ashby (1956), and which have been applied to the development of filamentous organisms (Lindenmayer, 1968). The Sordaria colony growing in a growth tube is treated as a filament of discrete segments which undergo changes of state in discrete time units according to the light or dark conditions to which they are exposed. The filament is considered as growing only at its apex, 6 hr of growth is arbitrarily chosen as the unit segment, and 6 hr intervals as the time units. The state of a segment in a given interval is determined by its state and its input (light or darkness) in the previous interval. The following states and inputs are assumed:

States: P—segment determined to produce perithecia, N—segment determined not to produce any perithecia, 1, 2, 3, 4—transitional dark-grown states, 5, 6, 7, 8—transitional light-grown states.

Inputs: L—light treatment, D—dark treatment.

The apical segment is assumed to give rise to another segment of equal length every 6 hr. In other words, we are assuming a constant rate of growth, independent of the light or dark input.

On the basis of the four assumptions listed in the Discussion for thioguanine-containing cultures, we construct the following transition matrix:

	1	2	3	4	5	6	7	8
L	65	N	8	7	65	7	8	N
D	21	3	4	P	N1	2	3	4

This transition matrix specifies a next state or pair of states for each intermediate state and each input. P and N are terminal states; once a segment is in one of these, it does not change any further. Where the value of the matrix is a pair of states, we are dealing with the growth of an apical segment. New segments always arise in states 1 or 5, depending on the input; these are the two initial states with which all computations must be started. We can perform sequential computations for any light-dark regime in which the alternating periods are in units of 6 hr.

Under continuous light or darkness the sequence of computed arrays will be very simple (the input shown next to the array is the one used to compute the next array):

L	5	D	1
L	6 5	D	2 1
L	7 6 5	D	3 2 1
L	8 7 6 5	D	4 3 2 1
L	N 8 7 6 5	D	P 4 3 2 1
L	N N 8 7 6 5	D	P P 4 3 2 1
	.	.	.	.
	.	.	.	.
	.	.	.	.

The progressions of intermediate states through which each segment passes may be thought of as processes of differentiation which result in 18 to 24 hr in the determination of a segment for bearing perithecia or staying vegetative.

The developmental sequence under alternating 24 hr periods of light and darkness is the following one:

```
L        5
L        6 5
L        7 6 5
L        8 7 6 5
D       N8 7 6  5
D       N4 3 2  N 1
D       NP4 3   N2 1
D       NPP4    N3 2 1
L       NPPP    N4 3 2   1
L       NPPP    N7 8 N   6 5
L       NPPP    N8 NN    7 6 5
L       NPPP    NNNN     8 7 6 5
D       NPPP    NNNN     N8 76  5
D       NPPP    NNNN     N4 3 2  N 1
D       NPPP    NNNN     NP4 3   N2 1
D       NPPP    NNNN     NPP4    N3 2 1
L       NPPP    NNNN     NPPP    N4 3 2   1
L       NPPP    NNNN     NPPP    N7 8 N   6 5
L       NPPP    NNNN     NPPP    N8 NN    7 6 5
L       NPPP    NNNN     NPPP    NNNN     8 7 6 5
        NPPP    NNNN     NPPP    NNNN     N 8 7 6 5
        L        D        L        D        L
```

The vertical lines separate the sections which originally developed in light or darkness (marked *L* or *D* at the bottom). These lines correspond to the boundaries which were marked on the growth tubes as the daily increments of growth. In agreement with Fig. 2, the perithecial zones appear at the later portions of light-grown sections, and the last perithecial zone is in the last-but-one light-grown section.

Similar computations can be performed for each of the light-dark regimes used for the experiments shown in Fig. 3, producing the corresponding zonation patterns. Only the last line of the computation corresponding to tube E is shown here (cultures grown for several days in the dark, exposed to 24 hr light, and returned to dark):

$$\ldots PPPPPNNN \mid NPPP \mid NPPPPP4321$$
$$\quad\quad D \quad\quad\quad L \quad\quad\quad D$$

It can also be demonstrated that light periods shorter than 24 hr produce shorter zones of inhibition preceding the boundary of the light-grown section.

The zonation patterns discussed so far could be constructed on the basis of purely verbal assumptions and arguments. But it would be considerably harder to figure out intuitively what the pattern should be for alternating periods of less than 24 hr duration, and for light and dark periods of unequal lengths. For example, what kind of zonation should we expect under an alternating 12 hr light-12 hr dark regime? The answer worked out with our model turns out to be:

$$NN \mid PN \mid NN \mid PN \mid NN \mid 8N \mid NN \mid 765$$
$$D \quad L \quad D \quad L \quad D \quad L \quad D \quad L$$

Narrow perithecial zones should develop following each dark-light boundary, and this is what was observed in a few experiments.

A similar question can be asked about alternating 6 hr periods of light and dark. The result obtained from the model is that no perithecial zones should develop, and that some of the segments will actually cycle through transitional states indefinitely, The lack of perithecial zones was confirmed by experiment.

This model is, of course, only one of many such constructions which would account for these observations. The fact that it is a discrete model makes the computations much easier to perform than with a continuous model based on differential equations. One important characteristic of this model is that no interactions need to be assumed to take place among the segments, while other processes can only be simulated by assuming such interactions.

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Characteristics of Two Lysine-independent Strains of *Streptococcus faecalis*

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SUMMARY

The ease of isolation of two mutants of *Streptococcus faecalis* 8043 appeared to be the result of the presence in the parent strain of a system for slow synthesis of lysine. The mutation to lysine independence occurred when the population became large enough for a significant number of spontaneous mutational events to occur. The slow growth and lysine depletion lysis of the parent strain selected for the new strain. The hydroxylysine-resistant mutant can arise from the parent strain or from the lysine-independent strain and is selected for by growth in a medium containing hydroxylysine. The three strains appeared to be identical by all tests employed except in their responses to lysine and hydroxylysine.

INTRODUCTION

It has been reported that *Streptococcus faecalis* ATCC 8043 has an absolute requirement for lysine (Dunn, Shankman, Camien & Block, 1947). The growth of this organism is inhibited by hydroxylysine despite the fact that it can utilize this lysine analogue for both protein and cell-wall synthesis in the presence of a sub-optimal concentration of lysine (Smith & Henderson, 1964). In view of these characteristics the strain has been given the designation Lys⁻OHLysˢ. The designation Lys⁻ or Lys⁺ is given to strains which are lysine-dependent or lysine-independent, respectively. Hydroxylysine-sensitivity and resistance are designated as OHLysˢ or OHLysʳ, respectively.

It was observed that a heavy inoculum of Lys⁻OHLysˢ added to either lysine-free medium or lysine-free medium containing hydroxylysine (3 to 4 μg./ml.) gave observable growth in each case after specified incubation periods (Smith & Henderson, 1964). Examination of cultures obtained under these conditions of growth revealed that they differed from each other and from the parent Lys⁻OHLysˢ strain with respect to their response to lysine and hydroxylysine. This communication describes the nature of these two variants which arose so readily from *Streptococcus faecalis* 8043.

METHODS

Streptococcus faecalis ATCC 8043 and two mutants whose isolation has been described (Smith & Henderson, 1964) were used. They were grown at 37° in a standard

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liquid medium (Henderson & Snell, 1948) which contained various amounts of L-lysine and/or the mixture of hydroxy-D,L-lysine and allohydroxy-D,L-lysine. Growth was measured at intervals by determining the extinction at 660 mμ with a Coleman Junior spectrophotometer.

The total viable count was determined by making the appropriate dilutions of the culture, shaking the diluted culture, with glass beads at each stage of dilution, and plating a sample, in triplicate, on 2 % agar plates prepared from standard-growth medium supplemented with lysine (100 μg./ml.). The plates were incubated at 37° for 24 hr. Total lysine-independent counts were made in a similar manner except that lysine-free medium was used. Numbers of lysine-dependent cocci represented the difference between the total viable count and the lysine-independent count.

Fermentation tests were conducted in standard growth medium in which glucose was replaced by the carbohydrate under test at an equimolar concentration (0·11 M). Standard growth medium either with 6·5 % NaCl or at pH 9·6 was sterilized by filtration for tolerance tests.

RESULTS

The isolations of the two mutant strains originally found by Smith & Henderson (1964) have been repeated under controlled conditions with a purified culture of the Lys⁻OHLys^s strain with the same results as previously obtained. The parent strain and the two mutants have been compared with respect to their performance in certain fermentation and tolerance tests. The results are presented in Table 1. The three strains fermented the same carbohydrates although not all carbohydrates to which *Strepto-*

Table 1. *Carbohydrate fermentation and response to various growth conditions of the three strains of Streptococcus faecalis*

Sugar	Lys⁻OHLys^s	Lys⁺OHLys^s	Lys⁺OHLys^r
	Carbohydrate fermentation		
Glucose	+ *	+ *	+ *
Maltose	+	+	+
Lactose	+ †	+ †	+ †
Mannitol	−	−	−
Sorbitol	−	−	−
Arabinose	−	−	−
Salicin	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
	Temperature of growth		
10°	+ †	+ †	+ †
37°	+	+	+
45°	+	+	+
	Special conditions		
Heated inoculum and incubation at 37°‡	−	−	−
Growth in 6·5% NaCl	−	−	−
Growth in pH 9·6	+ †	+ †	+ †

* A plus (+) represents growth, a minus (−) represents no growth.
† Growth was observed only after 3–5 days of incubation.
‡ The culture was heated at 60° for 10 min. in a water bath before it was used to inoculate fresh growth medium.

coccus faecalis is reported to respond (*Bergey's Manual of Determinative Bacteriology*, 7th ed., 1957) were utilized by these organisms. The apparent intolerance to 6·5 % NaCl was not expected. It has been proposed that *S. faecalis* 8043 (the Lys⁻OHLysˢ strain) be reclassified to *Streptococcus faecium* var. *durans* (Coultas, Albrecht & Hutchinson, 1060). The differences noted may reflect the characteristics of *S. faecium* (Diebel, Lake & Niven, 1963).

It has been shown (Dunn *et al.* 1947) that *Streptococcus faecalis* 8043 requires threonine, methionine, lysine and isoleucine for growth. In *Escherichia coli* (Umbarger & Davis, 1962) these four amino acids share certain common steps in their synthesis, i.e. the conversion of aspartic acid to aspartic semialdehyde (Black & Wright, 1955). Because of this relationship, also found in other bacteria, it was of interest to determine whether lysine-independence was accompanied by threonine- and methionine-independence.

Table 2. *Comparison of the number of lysine-independent bacteria in different samples of the same culture, and a series of different cultures*

	Expt. 1	Expt. 2.	
Number of cultures	50	100	1
Volume of cultures (ml.)	0·3	0·3	100
Number of samples tested	50	100	100
Volume of samples tested (ml.)	0·3	0·3	0·3
	Number of samples		
Number of lysine-independent bacteria			
0	14	31	0
1–10	4	1	0
11–20	0	7	0
21–30	1	15	7
31–40	2	13	42
41–50	6	8	47
51–100	4	15	4
101–300	1	10	0
> 300	18	0	0
Bacteria per culture	$9·4 \times 10^4$	$1·0 \times 10^5$	—
Mutation rate	$7·8 \times 10^{-5}$	$9·7 \times 10^{-5}$	—

The two mutant strains and the parent strain were grown in media containing various amounts of threonine or methionine. Both mutants had an absolute requirement for these amino acids. Thus lysine-independence is specific and does not result in the concomitant relief of threonine and methionine requirements.

The autolytic behaviour of the Lys⁻OHLysˢ strain has been previously demonstrated (Smith, Newman, Leach & Henderson, 1962) in various media. This strain is susceptible to lysine-depletion lysis as is *Streptococcus faecalis* 9790 (Shockman *et al.* 1961). The two mutant strains, Lys⁺OHLysˢ and Lys⁺OHLysʳ, are not subject to lysine depletion lysis (Smith & Henderson, 1964) because of their lysine biosynthetic capability; however, experiments conducted as previously described (Smith *et al.* 1962) indicated that the mutant strains did have lytic enzymes. Like the parent strain (Smith *et al.* 1962), the rate of lysis of the mutant strains was dependent on the suspending medium.

The rate of mutation to lysine-independence was determined by the fluctuation test (Luria & Delbrück, 1943). From the data presented in Table 2 it was calculated that

the rate was 7·8 to 9·7 × 10^{-5} mutations per cell per generation. The data further indicated that the acquisition of this trait did not result from the lack of lysine in the medium.
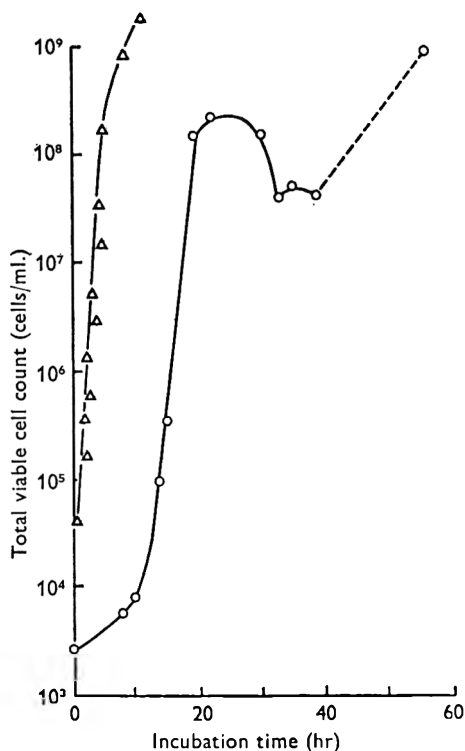
Fig. 1. Growth of the Lys$^-$OHLys^s strain in lysine-free and lysine-supplemented medium. The total viable count represents the average of triplicate samples. ($\triangle$—$\triangle$), growth in lysine-supplemented medium; ($\bigcirc$—$\bigcirc$), growth in lysine-free medium. The dashed line in the latter curve shows that between the 38th and 54th hr of incubation there was a substantial increase in viable cell count.

The mutation to lysine independence in *Streptococcus faecalis* was observed to occur with regularity after 90 hr under conditions where normal amounts of inoculum (10^3 washed cocci) were added to a medium which by independent assay with *Leuconostoc mesenteroides* was shown to be very low in lysine. Sub-culturing the growth revealed the presence of lysine-independent cells. In similar experiments, Lys$^+$OHLys^r cells appeared when Lys$^-$OHLys^s cells were grown in a lysine-free medium containing hydroxylysine 3 μg./ml. The 'leakiness' of the lysine synthetic pathway in the parent strain was established by two studies in which the growth rates were measured by making viable counts throughout the growth period. In the first experiment (Fig. 1) an inoculum of approximately 10^4 cocci from a log. phase culture of the Lys$^-$OHLys^s strain were added to 10 ml. of lysine-free medium or to 10 ml. of lysine-supplemented medium. The viable count was determined on lysine-supplemented agar medium. In the lysine-free medium the population did not change appreciably for about 4 hr, then logarithmic growth ensued at such a rate as to indicate a generation time of 37 min. In the lysine-supplemented medium growth was not delayed and the

generation time was 29·5 min. The maximum viable population was 10^9 cocci/ml. with lysine, but without lysine the maximum was 10^8 cocci/ml., and following a short stationary phase the viable population decreased. At 54 hr, however, Lys$^+$ OHLys cells were detected in the lysine-free medium when the viable population had reached 10^9 cocci/ml., suggesting that some time during the later phases of logarithmic growth mutants had appeared.

In another experiment viable Lys$^+$ cocci were also counted, beginning in the later stage of exponential growth and continuing for an additional 40 hr. This experiment, summarized in Fig. 2, showed that while viable Lys$^-$OHLys^s cells were disappearing the Lys$^+$OHLys^s strain was growing and that the latter strain emerged as the dominant
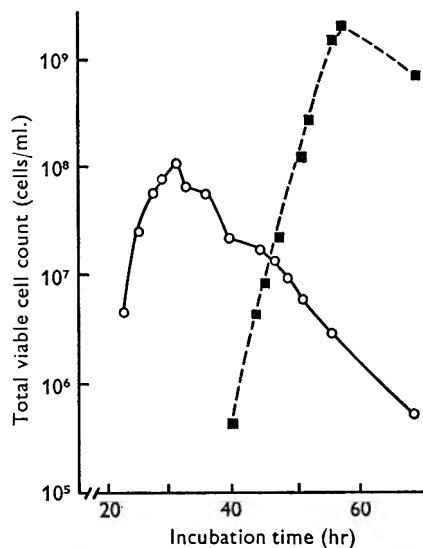
Fig. 2. Growth of the Lys$^-$OHLys^s strain in lysine-free medium and the emergence of the Lys$^+$OHLys^s strain as the dominant population. The experiment was conducted as described in the legend for Fig. 1 except that in addition the total viable lysine-independent cell count was made. (O—O), the lysine-dependent viable cell count; (■----■), the lysine-independent viable cell count.

population. The appearance of mutant cells in large numbers occurred when the population of Lys$^-$ cells was declining rapidly approximately 40 hr after inoculation. As in the previous experiment the mutant population continued to increase until it reached a maximum population of approximately 10^9 cocci/ml.

DISCUSSION

The parent and mutant strains have been compared and have been found to respond in the same way to all tests employed except for those previously described which involve the responses to lysine and hydroxylysine (Smith & Henderson, 1964). The present work has demonstrated that the characteristic of total lysine-independence resulted from a mutational event. Hydroxylysine-resistance has also been demonstrated to result from a mutational event involving the lysine-transport system (J. D. Friede, D. P. Gilboe and L. M. Henderson, to be published).

The Lys⁻OHLys^s strain has limited lysine biosynthetic capacity. In these experiments precautions were taken to assure that the medium was lysine-free and, in addition, a small inoculum was used. In the use of such an inoculum it was assumed that the growth rate for both parent and mutant was the same in lysine-supplemented medium and when a mutation occurred the lysine-dependent strain remained the dominant population as growth proceeded. In support of this assumption it was found that in an early stationary phase culture of Lys⁻OHLys^s cells in lysine-supplemented medium there were from 50 to 120 Lys⁺OHLys^s cocci/ml. in a total population of $4·3 \times 10^9$ cocci/ml. The use of a small inoculum, therefore, limited the possibility of a lysine-independent cell being present. This observation and the stability of the lysine-dependence trait of the parent organism which has been carried on lysine-containing medium suggests that the Lys⁻ strain has a selective advantage over the Lys⁺ strain under these culture conditions. Zamenhof & Eichhorn (1967) have presented evidence that mutants of *Bacillus subtilis* which have lost a biosynthetic function have a selective advantage over the parent strain. This observation provides an adequate explanation for the relative stability of the Lys⁻OHLys^s strain which has been carried in many laboratories over a period of years.

Despite the limited lysine biosynthetic capability of the Lys⁻OHLys^s strain certain pathway enzymes, aspartokinase, dihydrodipicolinic acid synthetase and diamino-pimelic acid decarboxylase, found in mutant strains, could not be detected in cell-free extracts of the parent strain (Gilboe, Friede & Henderson, 1968). Whether the lysine-independence of the mutant results from the relief of a pathway block or the dere-pression of part or all of the pathway has not been determined.

During the course of the growth of the Lys⁻OHLys^s strain in lysine-free medium a mutational event occurs resulting in lysine-independence. In earlier work (Dunn *et al.* 1947; Smith, 1963) growth of the parent strain in a rich lysine-free medium could not be detected nor was the outgrowth of the Lys⁺OHLys^s strain observed. The results of the 'fluctuation test' suggest that the lysine-free medium used in this work permitted limited growth of the Lys⁻OHLys³ strain and favoured the selection of the mutant strain which appeared.

In Fig. 1 and again in Fig. 2 it can be seen that in lysine-free medium the Lys⁻OHLys^s strain grew to a maximum viable population of only 10^8 cocci/ml. and, following a short stationary phase, the viable population decreased. It is postulated that the failure of Lys⁻OHLys^s cells to grow to a population of 10^9 cocci/ml. as it did in lysine-supplemented medium resulted from an imbalance between the rate of production of lysine, which is essential for protein and cell-wall synthesis, and lysis of the culture. If cell-wall subunit production were limited by lysine and the lytic system functions in the incorporation of subunits into the cell-wall mucopeptide (Shockman *et al.* 1961), then when no subunits are present lysis of the cell may ensue. It should be pointed out that when the population reached 10^8 cocci/ml. the pH value of the medium was found to have dropped to pH 6·0, the optimum for the lytic enzymes (unpublished observations).

The sequence of mutational events which convert the Lys⁻OHLys^s strain to the Lys⁺OHLys^s strain is not known. It has been shown, however, that Lys⁺OHLys^r cells can be isolated from cultures of the Lys⁺OHLys^s strain. Mutation to hydroxylysine resistance followed by mutation to lysine independence has not been excluded, but there is no obvious basis for selecting a Lys⁻OHLys^r strain.

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Transformation of Viridans-like Streptococci

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SUMMARY

Previous work has suggested that there are six divisions among the viridans-like streptococci of man. Reciprocal transformations were carried out between strains classified as *Streptococcus milleri* agg., *S. sanguis* agg. and *S. viridans* agg. The results of the quantitative transformation reactions confirmed a classification of these strains arrived at partly by traditional methods and partly by numerical methods.

INTRODUCTION

One method of assessing the validity of a proposed classification of bacteria is to compare genetic similarity between the strains allocated to one taxon with those allocated to other taxa. A classification of streptococci can be tested in this way at present by only three procedures: (1) the chemical or physical determination of deoxyribonucleic acid (DNA) base composition, (2) a measurement of the similarity between two groups of nucleic acid molecules in homology experiments where single-stranded nucleic acid molecules are mixed in conditions that allow them to re-form double strands, and (3) transformation experiments in which recombinants are produced by mixing cultures of bacteria with purified DNA.

The different species of streptococci do not show a large range of DNA base compositions (Pakula & Bankowska, 1964; Hill, 1966), so this method is not of use for testing the validity of proposed new species. It was not used here.

The assessment of relatedness by finding homology between nucleic acid molecules is, in the hands of Weissman and his co-workers (Weissman, Reich, Somerson & Cole, 1966), a precise method. These workers kindly examined a few of my strains. The results, given below, are in general agreement with the results of the transformation experiments reported here.

Transformation of streptococci (other than the pneumococci) was first observed by Bracco, Krauss, Roe & MacLeod (1957). Pakula, Hulanicka & Walczak (1958) extended the method and used the relative efficiency of transformation reactions as a measure of the closeness of relation between streptococci. Because recombination occurs in relatively few cells it is convenient to attempt to transfer a character for which a selective system is available. Transformation of antibiotic resistance is frequently used, and in the present study resistance to streptomycin was the character transferred.

The classification of the streptococci is far from settled. The enterococci and the pyogenic streptococci such as *Streptococcus pyogenes* are well classified, but many

strains isolated in hospital laboratories do not belong in either of these divisions and are frequently called viridans-like streptococci.

Studies of a collection of streptococci (Colman & Williams, 1965; Colman, 1967; Colman, 1968) have led to the conclusion that six divisions can be recognized among the viridans-like streptococci of man; namely *Streptococcus pneumoniae, S. salivarius, S. mutans, S. milleri, S. sanguis* and *S. viridans. S. pneumoniae, S. salivarius* and *S. mutans* are readily recognized and are homogeneous species. *Streptococcus mutans* (Clarke, 1924) is one of the limited range of bacteria that can cause dental caries in experimental animals and a collection of these organisms has been described by Edwardsson (1968). A few strains of this species, such as K I, were described in earlier papers in the present series. The three remaining divisions, *S. milleri, S. sanguis* and *S. viridans*, do not form equally clear-cut biological entities and can best be considered as aggregated species (agg.) from which subspecies can be made for special purposes. This paper reports the results of quantitative transformation reactions between members of the last three divisions.

METHODS

Strains. Thirty-seven strains were examined for transforming ability. They were selected as strains representative of a wide range of viridans-like streptococci. The cultures are maintained in the freeze-dried state and have been stored in this way since

Table 1. *Strains tested for transforming ability*

Strain	Name	Lancefield group	Strain	Name	Lancefield group
FW 73	milleri†	A	FW 213	sanguis	—
OG 7297	milleri	A	RB 1633*	sanguis	—
R 66/2884*	milleri	A	F 90 A*	sanguis	—
FW 102	milleri	C	MARTIN	uncertain	K
NCTC 8037	milleri	F	CHARWOMAN	uncertain	K
IS 14	milleri	F	NCTC 8031*	viridans	O
NCTC 5389*	milleri	F	FW 75*	viridans	O
IS 57*	milleri	F	FW 103*	viridans	O
RB 1634	milleri	G	FW 9	viridans	—
OG 7331	milleri	—	K 208	viridans	—
OG 7180*	milleri	—	NCTC 5338	viridans	—
SI 16*	milleri	—	BU 174	viridans	—
NCTC 8606	salivarius	K	NCTC 3858	uberis	E
BU 104	salivarius	K	NCTC 7466	pneumoniae	—
NCTC 7366	salivarius	—	NCTC 8181	agalactiae	B
NCTC 7868*	sanguis	H	HS 6	mutans	—
FW 229*	sanguis	H	K I	mutans	—
NCTC 3165*	sanguis	—	10987	mutans	—
FW 225	sanguis	—			

* Competent strains.
† The epithets 'milleri', 'sanguis' and 'viridans' are the names of the proposed aggregated species.

they have been isolated or received from culture collections. The strains are listed in Table 1, which also shows how the strains were classified before the transformation experiments were started. Twenty-nine of these strains were included in a previous study (Colman, 1968) where this manner of classification was proposed. It must be emphasized that the strains with the Lancefield group A antigen do not have the physiological or biochemical properties of *Streptococcus pyogenes*.

Preparation of DNA. Mutants resistant to streptomycin 1 mg./ml. were isolated from 37 strains. DNA was prepared from each of these mutants by the method of Kirby, Fox-Carter & Guest (1967). The following four procedures were the modifications made to the method. The bacteria were cultured in Tryptone Soya Broth (Oxoid) supplemented with 0·3 % Yeast Extract Powder (Oxoid). The cultures were incubated at 32° for about 30 hr. The bacteria were disrupted by vibration in a Mickle Tissue Disintegrator (Bracco *et al.* 1957) for 10 min. with Ballotini grade-12 glass beads. DNA was precipitated from the aqueous phase by adding an equal volume of 2-ethoxyethanol; sodium benzoate and *m*-cresol were not used.

The amount of DNA in the preparation was estimated by the method of Burton (1956), care being taken that equal amounts of NaCl were present in all tubes.

Medium. The medium used for transformation experiments contained the ingredients of the second medium (ET3) of Pakula & Walczak (1963). The complete medium was adsorbed with activated charcoal by the method of MacLeod & Mirick (1942). Horse serum, previously heated at 62° for 30 min., was always added (10 %, v/v) to the medium just before use.

Testing of strains for competence. Strains to be tested for the ability to incorporate DNA in the transformation reaction were grown overnight in ET3 medium at 32°, and 1 % (v/v) of this culture was added to warmed ET3 medium. The subculture was incubated for 1 hr at 37°, and then a sample was removed and the chains disrupted with an ultrasonic disintegrator (MSE, model 100 W, 20 kcyc./sec., 6 μ amplitude, for 1 min.). A sample was diluted 1/10 in warmed medium and 2·5 ml. of the diluted culture added to each of three tubes standing in a water bath at 37°. One tube contained 0·5 ml. M-NaCl, the second 30 μg. of DNA from the variant culture of the test strain, and the third tube 30 μg. of DNA from a mutant of strain CHALLIS (NCTC 7868). To ensure that the DNA solutions were biologically active a wild-type culture of strain CHALLIS, prepared in a similar way, was incubated in a second and similar row of tubes. The strain CHALLIS is known to be an efficient acceptor of DNA in the transformation reaction (Pakula & Walczak, 1963).

After incubation for 15 min., 10 μg. of deoxyribonuclease in 2 ml. 0·15 M-NaCl was added to destroy any remaining extracellular DNA. A series of ten-fold dilutions was then prepared from each tube and 1 ml. samples from each dilution and from the undiluted medium taken for culture in roll-tubes. The Astell roll-tube apparatus was used for these cultures and the growth medium was Milk Agar (Oxoid) supplemented with 1 % Neopeptone (Difco). The roll-tubes containing the higher dilutions were used for viable counts. The roll-tubes containing the lower dilutions and the undiluted medium were incubated for 2 hr at 37°, and then a further 2 ml. of Milk Agar containing streptomycin 1 mg./ml. was layered in the roll-tube. Any colonies that developed were counted after 2 days' incubation at 37°.

Streptococci are not equally competent throughout the growth cycle (Pakula & Walczak, 1963). Strains were therefore tested for competence at 1 hr after subculture and at 0·5 hr intervals after that for a further 3 hr if the strain grew rapidly or 4 hr if the strain grew more slowly.

Reciprocal transformation experiments. The protocol for quantitative reciprocal transformation experiments was similar to that of the screening experiments. The screening experiments just described provided information as to whether the strain could incorporate DNA, and if so, when the greatest proportion of competent cocci

would be found in a culture. Fresh DNA preparations were made from mutants of cultures that were transformed.

The test strain was subcultured for the expected optimal period and then exposed to the new DNA preparations from mutants of each of the competent strains. Each strain was tested at least three times and the experiments were done on different days, but with the same DNA preparations.

RESULTS AND DISCUSSION

Cultures transformed

In the first series of experiments 13 of the 37 strains were transformed by DNA from streptomycin-resistant strains. These strains were: OG7180, IS57, NCTC5389, NCTC7868, FW229, NCTC3165, NCTC8031, FW75, FW103, S116, RB1633, R66/2884 and F90A. These strains are all members of one or other of the proposed aggregated species *Streptococcus milleri*, *S. sanguis* and *S. viridans*.

Perry & Slade (1962) also found a restricted distribution of transformable streptococci. They described their strains solely on serological criteria but their strain C628 was physiologically similar to *Streptococcus milleri*. Their group H strains are probably similar to *S. sanguis* and one strain NCTC7868 (CHALLIS) was used in both studies. Their strains of serological group O are probably similar to the streptococci referred to as *S. viridans*.

Five of the 13 strains could be transformed regularly and with relatively high frequency. The strains and the highest rates of transformation achieved were: NCTC 7868 (3 %), NCTC3165 (0·01 %), F90A (0·01 %), IS57 (0·7 %), and FW75 (0·1 %). One recipient, NCTC8031, classified as *Streptococcus viridans*, grew many recombinants in some experiments and few in others. When many cocci were transformed in cultures of NCTC8031 then the number of recombinants produced by the various DNA preparations had the same relative proportions as found with strain FW75, also a strain of *S. viridans*.

Reciprocal transformation

The 13 wild-type cultures that had been transformed in the first series of experiments were subjected to reciprocal transformation. In each reciprocal transformation experiment a wild-type recipient was exposed to preparations of DNA from 13 streptomycin-resistant variants. Two DNA preparations were used from the variant of the strain F90A and therefore each recipient strain was exposed to a total of 14 DNA preparations.

When transformation experiments were repeated with new DNA preparations three strains, OG7180, FW229 and RB1633, which had been transformed in the first series of experiments already described, failed to show any transformation with the new DNA preparations; this difference could not be explained.

Table 2 lists the numbers of transformants in three experiments. The three strains tested are all members of the aggregated species *Streptococcus milleri*. These experiments show that cultures of *S. milleri* can incorporate DNA from strains of the three species *milleri*, *viridans* and *sanguis*; thus the proposed species is not genetically isolated.

The first recipient in Table 2, IS57, was transformed with high frequency and the other two recipients, R66/2884 and NCTC5389, were transformed with low frequency.

Table 2. *Comparison of the numbers of transformants produced in three experiments*

One recipient strain, IS57, was an efficient acceptor of DNA and this strain showed large differences between the numbers of transformants produced in intra-specific and inter-specific transformations

DNA preparations	Recipient strains (*S. milleri*)		
	IS57	R66/2884	NCTC5389
S. milleri agg.			
IS57	456	15	15
R66/2884	46	9	2
NCTC5389	49	1	18
OG7180	102	6	4
S116	231	0	9
S. viridans agg.			
FW75	1	0	0
FW103	3	13	3
NCTC8031	2	0	0
S. sanguis agg.			
NCTC7868	4	1	1
F90A (a)	2	1	0
F90A (b)	1	0	1
FW229	4	0	0
NCTC3165	2	0	1
RB1633	10	8	0
NaCl	0	0	0
Mean viable count	$6 \cdot 09 \times 10^6$	$2 \cdot 05 \times 10^6$	$1 \cdot 43 \times 10^6$

A column lists the numbers of transformants produced in one experiment in which streptomycin-resistant recombinants were produced by exposing a strain to DNA from resistant mutants.

Table 3. *Numbers of transformants produced in three experiments*

In each experiment an efficient acceptor strain of a different species was used

DNA preparations	Recipient strains		
	S. sanguis NCTC7868	*S. milleri* IS57	*S. viridans* FW75
S. sanguis agg.			
NCTC7868	5100	181	77
F90A (a)	1120	260	87
F90A (b)	1380	122	72
FW229	720	90	62
NCTC3165	790	110	61
RB1633	49	96	80
S. milleri agg.			
IS57	133	13400	71
R66/2884	22	1610	28
NCTC5389	22	1800	28
OG7180	78	4100	48
S116	123	8300	60
S. viridans agg.			
FW75	70	74	318
FW103	108	111	900
NCTC8031	73	55	640
NaCl	0	0	0
Mean viable count	$1 \cdot 77 \times 10^6$	$1 \cdot 51 \times 10^7$	$6 \cdot 20 \times 10^5$

A column lists the numbers of transformants produced in one experiment in which streptomycin-resistant recombinants were produced by exposing a strain to DNA from resistant mutants. Two DNA preparations were used from strain F90A.

In these experiments the strain that was transformed with high frequency produced more recombinants in intra-specific transformation than in inter-specific transformation. All strains used had been classified before the transformation experiments were begun. The cultures that were transformed with low frequency did not show large differences in the relative efficiency of transformation by DNA from different species. In the measurement of genetic similarity by the transformation reaction it is
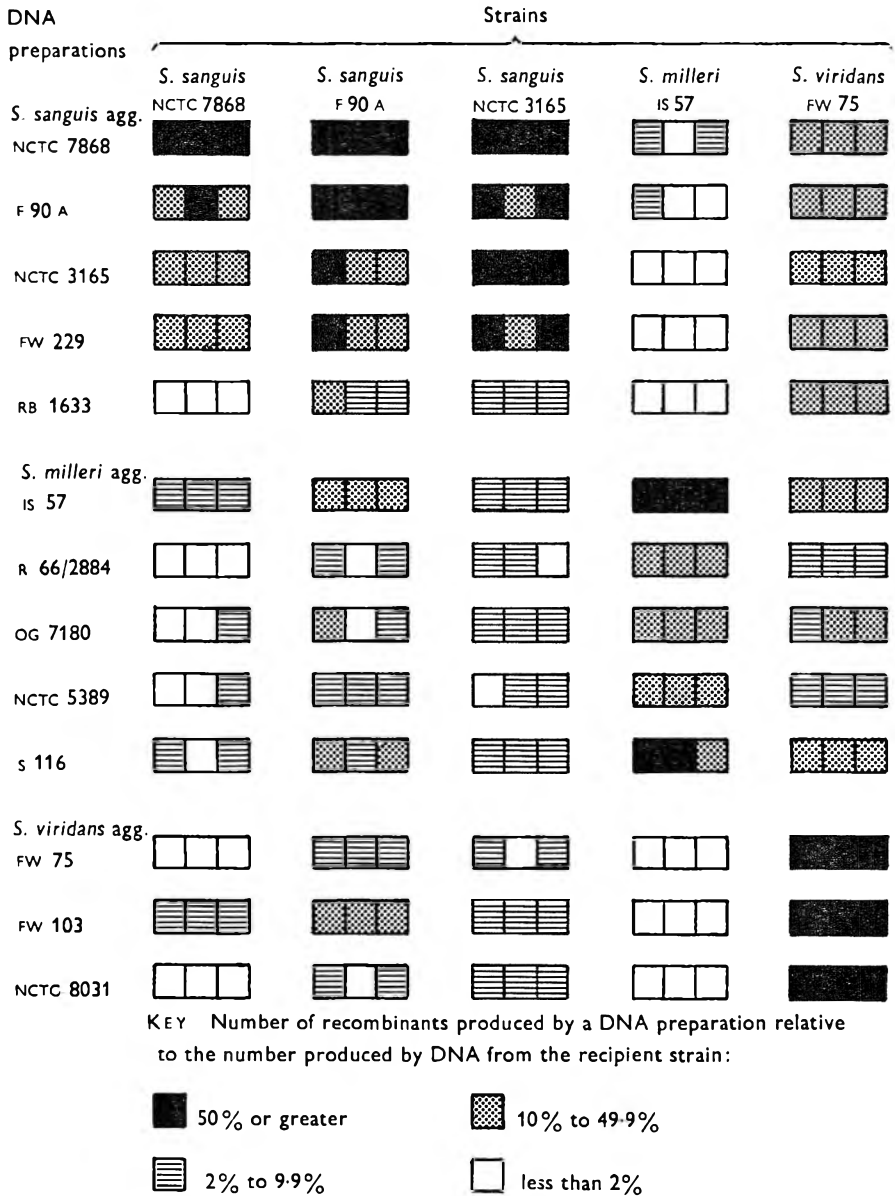

Fig. 1. Transformation between three species. Results with strains that are transformed with high frequency. DNA was extracted from streptomycin-resistant mutants and mixed with wild-type strains.

therefore necessary to use a quantitative reaction as opposed to a qualitative, and the recipient strain should be capable of being transformed at a reasonably high efficiency by suitable DNA.

Table 3 shows the results of three reciprocal transformation experiments with strains that are efficient acceptors of DNA in intra-specific transformations. The numbers of transformants are listed because the number of recombinants in intra-specific transformation is large and the number in inter-specific transformation is small and detail is lost by representation of the data in a histogram: the range in counts is from 13400 to 22.

The results of three consecutive experiments with each of the five strains that transform with high efficiency are brought together in Fig. 1. Here the results are shown as percentages of the proportion of cocci transformed by DNA from a mutant of the recipient strain. Thus, the proportion of cocci in a culture of NCTC 7868 transformed by DNA from a streptomycin-resistant variant of the same strain is taken as 100 %. Figure 1 taken overall shows that transfer between strains classified in one species was more efficient than between strains in different species.

The concept of a species as being composed of, and limited to, those individuals who can contribute to a gene pool is of value in higher plants and animals. It is of little value in defining the bacterial species because the mechanisms of genetic exchange are simple. Genetic exchange can occur between dissimilar bacteria, for example between members of the genera *Escherichia*, and *Shigella*, *Salmonella* and *Serratia* (Hayes, 1964), but it is not convenient to classify these bacteria as one species.

Genetic homology experiments

Dr Sherman M. Weissman and his colleagues (R. M. Cole, personal communication) tested six strains for genetic homology by the method of Reich, Somerson, Rose & Weissman (1966). Radioactive ribonucleic acid (RNA) complementary to

Table 4. *Relatedness values of the streptococci in the genetic homology experiments carried out by Dr S. M. Weissman and Dr R. M. Cole*

These workers have suggested that a value of 0·10 to 0·20 or greater is characteristic of a species

	IS57 *S. milleri,* group F indifferent	FW73 *S. milleri,* group A indifferent	BU104 *S. salivarius,* group K indifferent	Kjems' strain *S. pyogenes,* group A haemolytic	F90A *S. sanguis,* — haemolytic
FW73	0·28	—	—	—	—
BU104	0·20	0·97	—	—	—
Kjems' strain	$4·2 \times 10^{-5}$	$3·1 \times 10^{-6}$	$3·3 \times 10^{-5}$	—	—
F90A	$1·1 \times 10^{-3}$	$3·4 \times 10^{-5}$	$3·9 \times 10^{-4}$	$1·8 \times 10^{-5}$	—
NCTC 7868 *S. sanguis,* group H haemolytic	$7·8 \times 10^{-4}$	$5·5 \times 10^{-5}$	$2·8 \times 10^{-4}$	$8·5 \times 10^{-3}$	0·31

DNA was synthesized. The RNA was incubated with DNA and any DNA-RNA hybrids were retained on a filter and the radioactivity measured. Weissman *et al.* (1966) found the method sufficiently precise to distinguish serotypes of *Streptococcus pyogenes.*

Five of the six strains examined were also tested in the transformation system: FW73, IS57, BU104, NCTC 7868 and F90A, but two of these, FW73 (a strain of *Strepto-*

coccus milleri carrying the Lancefield A antigen) and BU104 (*S. salivarius*, Lancefield group K), could not be transformed. The sixth strain was Kjems' strain of *S. pyogenes* (Lancefield group A) 61×101 used by Weissman *et al.* (1966) in their studies of streptococci. The results of Weissman and Cole's experiments with the six strains are shown in Table 4 and these results are published with their permission. The relatedness value of two strains was obtained by calculating the product of the logarithms of the counts of radioactivity in the two heterologous DNA-RNA reactions and dividing it by the product of the logarithms of the counts in the two homologous reactions.

The two strains reacting as serological group A, one *Streptococcus milleri* FW73 and the other *S. pyogenes* 61×101, were shown in the homology experiments to be only remotely related to one another, but the indifferent strain FW73 had considerable genetic similarity to IS57, an indifferent strain of Lancefield group F. FW73 showed even greater homology with the strain of *S. salivarius* BU104. The genetic homology between FW73, IS57 and BU104 was greater than that between *S. pyogenes* and strains of Lancefield group C that can be presumed to be *S. equisimilis* (Weissman *et al.* 1966). These three strains were also clustered together at 80 % overall similarity in computer studies (Colman, 1968). The strains of *S. sanguis*, NCTC7868 and F90A, showed considerable genetic homology with each other but very little with the other four strains.

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Competence in *Haemophilus influenzae*.
A Role for Inosine and Lactate in the Primary Cell-deoxyribonucleic Acid Attachment Reaction

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SUMMARY

Cells of *Haemophilus influenzae* grown with or without competence promoting factors, inosine and lactate, were compared in their ability to bind deoxyribonucleic acid (DNA) and to undergo a genetic transformation. It was found that cells grown with the above factors bound 70 % of the ^{14}C-labelled DNA and transformed to a relatively high level (1·4 to 2·5 %). Cells grown without the factors bound only 8 % of the ^{14}C-labelled DNA and transformed to a low level (0·01 % or less).

It is suggested from these studies that during growth cells of *Haemophilus influenzae* use inosine and lactate either to form or modify that portion of the cell surface that can later become involved in binding DNA.

INTRODUCTION

Ranhand & Herriott (1966) reported that the development of competence in *Haemophilus influenzae* was dependent on the presence of both inosine and lactate during growth (phase 1). It was unknown at that time whether these factors influenced the primary cell-DNA reaction or the recombinational events that follow the uptake of DNA. This report shows, by comparing directly the extent of transformation and the amount of ^{14}C-labelled DNA bound by cells grown with or without inosine and lactate, that it is the primary cell-DNA reaction that is affected.

METHODS

Organism and transforming DNA. Haemophilus influenzae strain RD and the method used for preparing transforming DNA from cells that were resistant to more than streptomycin 2 mg./ml. were described by Goodgal & Herriott (1961). DNA derived from cells that were resistant to novobiocin (cathomycin) 25 μg./ml. was a gift from Dr R. M. Herriott, Johns Hopkins University, Baltimore, Md.

Phase 1 growth medium and cultural conditions. The trypticase growth medium containing glucose (TCMG) and the cultural conditions used were as previously described (Ranhand & Lichstein, 1966). In one experiment (Table 2) glucose was deleted from the growth medium (TCM). When glucose, lactate and inosine were

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deleted from the medium, adenosine 660 μg./ml. was added. This addition was found necessary for growth.

Phase 2 medium for the development of competence and transformation procedures. The phase 2 medium used for the development of competence was essentially that of Spencer & Herriott (1965) modified with 0·05 % acid hydrolysed casein (Ranhand & Lichstein, 1966). The transformation procedures used were as described by Ranhand & Lichstein (1966).

^{14}C-labelled DNA. ^{14}C-labelled DNA was prepared from cells grown in TCM containing inosine, 1·32 mg./ml., lactate 660 μg./ml. and thymidine-2-^{14}C 0·05 μc./ml. by methods already described (Goodgal & Herriott, 1961). The thymidine had a specific activity of 30 mc./mmole and was obtained from the New England Nuclear Corp., Boston, Mass. The high inosine concentration, suggested by Dr R. M. Herriott, allows for more efficient utilization of the thymidine. The final DNA preparation had an approximate specific activity of 6×10^3 c.p.m./μg. and was used without prior dialysis.

Radioactivity determinations. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer model No. 3003 using Bray's solution (1960). Background was subtracted from the values presented.

RESULTS

Kinetics of the development of competence. Figure 1 shows the kinetics of the development of competence, measured by transformation to streptomycin resistance, by cultures of *Haemophilus influenzae* grown in TCMG with or without inosine and lactate. Cells grown with the factors developed competence rapidly and reached a maximum value of 2·5 % at about 100 min. (The per cent transformation equals the number of transformed cells/ml. × 100 divided by the number of viable cells/ml.) Cells grown without inosine and lactate did not develop competence appreciably over the 100 min. tested and transformed to a maximum value of 0·01 %. In both cultures the viable count increased slightly.

Although not shown, a similar result was obtained when a completely defined medium described by Ranhand (1964) was used for phase 1.

Effect of inosine and lactate on DNA binding to cells. To achieve successful transformation at least two steps must occur: (i) the attachment of DNA to the cells and (ii) the incorporation of the DNA into the resident genome. Because the results presented in Fig. 1 did not reveal why cells grown without inosine and lactate did not transform, two experiments were done to determine which of the above steps was influenced by inosine and lactate.

The first experiment was designed to show the amount of DNA taken up by cells by measuring the amount of residual (unreacted) DNA left in the supernatant fluid. If cells grown without inosine and lactate were capable of taking up DNA, for example, they were competent but not able to integrate the DNA into their genome, then most, if not all, of the DNA added should have been removed from the reaction mixtures under the conditions described below. On the other hand, if the cells could not take up DNA, then the unreacted DNA should have been available for transforming other fully competent cells.

Cells were grown with or without inosine and lactate and were put through a

competence development procedure as previously described (Ranhand & Lichstein, 1966). A limiting amount of DNA (1×10^{-3} μg./ml., determined independently) carrying the streptomycin resistance marker was added to these cells and transformation to streptomycin resistance was determined in the usual manner. After removing a test sample (0·1 ml.), competent cells that were already resistant to novobiocin were
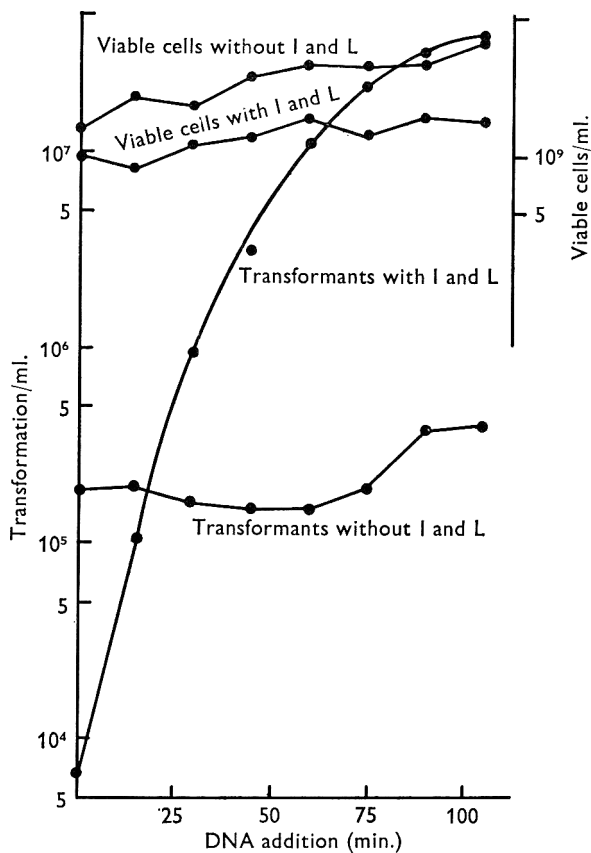
Fig. 1. Kinetics of the development of competence. Cells were grown with or without inosine (I) 330 μg./ml. and lactate (L) 330 μg./ml. in TCMG and diluted into the competence medium (Ranhand & Lichstein, 1966). At the indicated times, DNA 1 μg. carrying the streptomycin-resistant marker and 8-azaguanine 5 μg. were added/ml. The cells were shaken for 30 min. after the additions, at which time they were diluted and plated as described previously (Ranhand & Lichstein, 1966).

added to the reaction mixtures and residual DNA carrying the streptomycin marker was assayed by observing the number of cells resistant to both streptomycin and novobiocin. The use of novobiocin-resistant competent cells for the second transformation reaction eliminated a potentially large background of streptomycin-resistant cells. In other experiments, samples were heated to 60° for 30 min. before assaying the residual DNA, and the results obtained were similar. Furthermore, analogous results were also obtained when the defined medium described by Ranhand (1964) was used for phase 1.

The results of this experiment (Table 1) show that cells grown with inosine and lactate removed almost 100 % of the input DNA, indicated by the low level of streptomycin novobiocin-resistant cells obtained (column 5), whereas cells grown without inosine and lactate removed only 5 %. These values are based on the number of transformations/ml. to streptomycin resistance observed (2·1 × 10⁴/ml.) using competent novobiocin-resistant cells and DNA 1 × 10⁻³ μg./ml.

Table 1. *The effect of inosine and lactate on DNA uptake*

Supplements to TCMG	DNA (μg./ml)	Transformations /ml. to *str-r* × 10⁻⁵	Viable cells/ml. × 10⁻⁹	Transformations /ml. to *str-r* among *nov-r* cells	Transformation* (%)	Residual DNA (*str-r*) (%)
Inosine and lactate	10	150	1·1	—	1·4	—
Inosine and lactate	10⁻³	0·44	2·0	50	—	0·24
None	10	1·7	2·2	—	0·01	—
None	10⁻³	0·028	2·4	2 × 10⁴	—	95

Cells were grown in a trypticase medium containing glucose (TCMG) with or without inosine 330 μg/ml. and lactate 330 μg./ml. and were put through a competence development procedure as previously described (Ranhand & Lichstein, 1966). DNA was added at the indicated concentrations for 30 min., at which time 0·1 ml. of culture was used to assay the number of transformations to streptomycin resistance (*str-r*). The remainder of the reaction mixtures were kept at 4°, and on the following day competent novobiocin-resistant (*nov-r*) cells were added to them. Residual DNA was assayed by observing the number of cells that were both *nov-r* and *str-r* using standard procedures. The control consisted of competent *nov-r* cells and *str-r* DNA 10⁻³ μg./ml., which gave 2·1 × 10⁴ *str-r* transformants/ml. This value was taken as the 100 % value to calculate the residual DNA present in column seven.

* Number of transformed colonies/ml. × 100 divided by number of viable cells/ml.

Table 2. *The uptake of* ¹⁴*C-labelled DNA by Haemophilus influenzae grown with or without inosine and lactate*

Supplements to TCM	DNA addition (min.)	DNAase (5 μg./ml.)	% radioactivity Supernatant	Cells
None	0	—	87	12
None	0	+	97	3
Inosine and lactate	0	—	91	9
Inosine and lactate	0	+	99	1
None	80	—	92	8
None	80	+	97	3
Inosine and lactate	80	—	29	71
Inosine and lactate	80	+	68	32

Cells were grown in a trypticase medium minus glucose (TCM) with or without inosine 660 μg./ml. and lactate 660 μg./ml. (Methods) and treated as described previously (Ranhand & Lichstein, 1966). At zero-time, cells were diluted into the phase 2 medium containing 8-azaguanine 5 μg./ml. (Ranhand & Lichstein, 1969) and ¹⁴C-labelled DNA 0·05 ml. (0·066 μg; 342 c.p.m.). They were shaken for 30 min. after the addition of the DNA, and pancreatic deoxyribonuclease (DNAse) was added when required for 5 min. The cells were centrifuged, and the supernatant fluids were removed. Cell pellets were resuspended in 1·0 ml. of water. Appropriate samples were counted in the liquid scintillation spectrometer. The same procedure was followed at 80 min.

The second experiment was designed to test whether cells grown without inosine and lactate bound any DNA at all. ¹⁴C-labelled DNA was added to cells grown with or without inosine and lactate at zero-time and at 80 min. in phase 2. The results (Table 2) show that both cultures reacted poorly with DNA at zero-time. At 80 min., however,
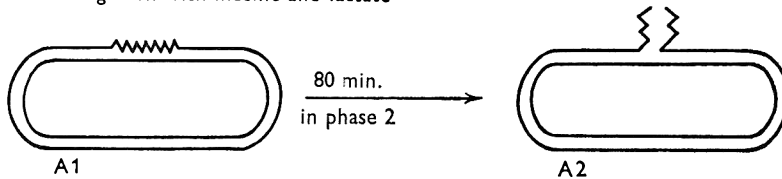
only cells grown with inosine and lactate reacted with DNA and adsorbed about 70 %
of it. On the other hand, cells grown without inosine and lactate adsorbed only 8 %.
Furthermore, only cells grown with the factors bound an appreciable amount of DNA
irreversibly (45 %) as determined by its resistance to pancreatic deoxyribonuclease.

The results obtained from these two experiments clearly showed that inosine and
lactate did indeed influence the binding of DNA to cells. However, the role, if
any played by inosine and lactate on the recombination events remains to be found.

DISCUSSION

In an earlier report, Ranhand & Lichstein (1966) suggested that inosine and lactate
were functioning at or near the DNA binding site. In addition, they showed that these
sites could be *equally* destroyed by periodate when cells were treated either at zero-
time (low competence) or at 80 min. (high competence) in phase 2. This result was
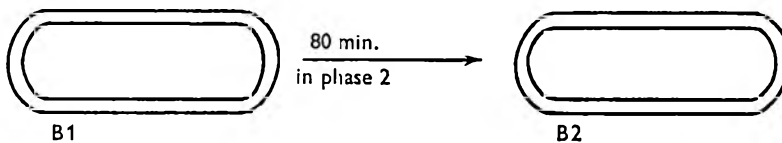
Fig. 2. A model comparing *Haemophilus influenzae* cells grown with or without inosine and
lactate. A1: Premature DNA binding site presumably derived from the metabolism of
inosine and lactate during growth is present but it cannot yet react with DNA; however, it
can be inactivated by periodate (Ranhand & Lichstein, 1966). There are about 27 such sites
per cell (Barnhart & Herriott, 1963). A2: After 80 min. in phase 2 the premature site
becomes modified so that it can now react with DNA. This modification appears to be
dependent on protein synthesis (Ranhand & Lichstein, 1969). These sites can also be destroyed
with periodate. B1: Premature DNA binding site not present due to absence of inosine and
lactate during growth. B2: No DNA sites present; therefore, cells are non-competent.

interpreted to mean that inosine and lactate contributed to a site (presumably on the
cell surface) that later became modified into a DNA binding site upon further incuba-
tion in phase 2. The results presented in Table 2 may further support this hypothesis
because at zero-time in phase 2 cells grown with or without inosine and lactate do not
bind large amounts of DNA.

Although the exact role played by inosine and lactate is as yet unknown, an inter-
pretation of the data is presented diagrammatically in Fig. 2. Cells grown with inosine

and lactate have a site(s) on their surface that is important for the development of competence but it is not reactive towards DNA at zero-time in phase 2, that is, it cannot bind DNA (Fig. 2 A1). Incubation for 80 min. in phase 2, however, causes the modification of these pre-existing sites into DNA binding sites (Fig. 2 A2) presumably by processes that require protein synthesis (Ranhand & Lichstein, 1969). Cells grown without inosine and lactate lack these sites or modifications (Fig. 2 B1), and therefore cannot react with DNA at 80 min. in phase 2 (Fig. 2 B2).

Unfortunately, studies made with lactate-U-^{14}C and inosine-8-^{14}C have not yet revealed their specific functions.

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Susceptibility and Resistance of Various Strains of *Mycoplasma hyorhinis* to Antisera, Polymyxins and Low pH Values

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SUMMARY

The growth of some strains of *Mycoplasma hyorhinis* on solid medium was inhibited by rabbit antisera incorporated in filter-paper discs. Other strains were resistant to the same antisera. Rings of precipitate were observed in the agar around the discs. The largest number of precipitation rings and the most intense ones occurred with antiserum-sensitive strains. The antiserum-sensitive strains were also more sensitive than the resistant strains to colistin and polymyxin B when these antibiotics were incorporated in paper discs. Colonies of antiserum-resistant strains developed on solid medium containing low concentrations of horse serum, while colonies of sensitive strains developed only on solid medium containing higher concentrations of serum. The growth of antiserum-sensitive strains, but not resistant ones, was suppressed on solid medium at pH 6·5. All these differences between sensitive and resistant strains were only expressed on solid medium; the phenomena were not observed when the organisms were grown in liquid medium. It is suggested that the differences between sensitive and resistant strains are due to changes in the composition of the mycoplasma membranes.

INTRODUCTION

Mycoplasma hyorhinis (Switzer, 1955) may be identified by various serological tests. However, in disc growth-inhibition tests on agar medium (Clyde, 1964) some strains were not inhibited by antisera against *M. hyorhinis* (Dinter, Danielsson & Bakos, 1965). In similar tests, Hayflick & Stanbridge (1967) also observed resistance to antibodies and ascribed this to antigenic shifts occurring in organisms growing on agar medium. We have examined in greater detail the resistance and susceptibility to anti-*M. hyorhinis* sera of various strains of *M. hyorhinis* and have observed other biological phenomena with the same strains. The results show that these phenomena occur only when the organisms are grown on solid media and not when they are grown in liquid media.

METHODS

Mycoplasma media. The standard liquid medium consisted of beef-heart infusion with addition of 10 % (v/v) extract of baker's yeast, 1 % (w/v) glucose, 20 % (v/v) unheated horse serum, penicillin (100 u./ml.), 0·05 % (w/v) thallium acetate and

0·002 % (w/v) phenol red, adjusted to pH 7·8. The extract of baker's yeast was prepared according to the method of Hayflick (1965). The standard agar medium was composed of the same ingredients except that glucose was decreased to 0·1% and phenol red omitted; the agar (Noble, Difco) was 1 % (w/v), and the medium was adjusted to pH 7·5. The agar medium, poured in plastic Petri dishes, was used within 1 to 3 days after being prepared.

Mycoplasma strains. Nine strains of *Mycoplasma hyorhinis* were studied. Five of them (F, S7, 200, SK, G) were used in a previous study (Dinter *et al.* 1965). Four other strains (F/D, K, GDL, SWITZER 9832, here called 'W') were added to this group. F/D is an antiserum-insensitive derivative of the F strain. The strains W, K and GDL were kindly supplied by Drs D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent, England), K. Bakos (National Veterinary Institute, Stockholm, Sweden) and R. H. Purcell (National Institutes of Health, Bethesda, U.S.A.), respectively. Stock cultures of the strains in liquid medium were kept at −60°.

Cultivation. All cultures were incubated at 37°, agar cultures being placed in containers with moist air.

Millipore filtration. Liquid cultures were filtered through membranes (Millipore Filter Co., Bedford, Mass., U.S.A.), of average pore diameter 650 mμ, 450 mμ, 300 mμ and 220 mμ, by using a Swinny adaptor. The number of organisms before and after each filtration was measured.

Antibiotics. The following preparations of polymyxin were used: polymyxin B (Novo Industri, Copenhagen) and colistin sodium methanesulphonate (H. Lundbeck & Co., Copenhagen-Valby, Denmark). These antibiotics were diluted in liquid medium of pH 7·8 to produce concentrations indicated in Tables 5 and 6. Paper discs (6 mm. diameter) containing various antibiotics (Department of Bacteriology, Karolinska Sjukhuset, Stockholm) were also used. The following were used, with the amounts/disc indicated in parentheses: bacitracin (10 i.u.), chloromycetin (30 μg.), colistin sodium methanesulphonate (900 i.u.), erythromycin (50 μg.), gentamycin (10 μg.), kanamycin (5 μg.), neomycin (50 μg.), novobiocin (50 μg.), polymyxin B (50 μg.), streptomycin (50 μg.), terramycin (50 μg.), tetracycline (50 μg.), vibramycin (50 μg.).

Rabbit antisera. Antisera to 8 strains of *Mycoplasma hyorhinis* were available, some of them having been used in a previous study (Dinter *et al.* 1965). These antisera, stored at −20°, were used after being heated for 30 min. at 56° and absorbed with a 10-fold concentrate of liquid medium as described by Dinter *et al.* (1965).

Gel diffusion. Antigen production and the microtechnique were as described previously (Dinter *et al.* 1965).

Metabolic inhibition (m.i.). For tests with antisera this test was done by a microtechnique as described previously (Taylor-Robinson, Purcell, Wong & Chanock, 1966). The results were recorded when a colour change equivalent to about half a pH unit had occurred in the wells containing mycoplasma organisms but no antiserum. In tests with colistin, the technique was modified and was done in tubes with 1 ml. medium/tube. Each mycoplasma strain was diluted in serial 10-fold steps in the presence or absence of various amounts of colistin in the medium. The results were read when the colour change in the titration series without colistin had ceased to progress. For both types of test one colour-changing unit (c.c.u.) of mycoplasma activity was defined as the highest dilution of organisms which produced a colour change.

Disc growth-inhibition. The technique was that of Clyde (1964). Stock mycoplasma cultures, previously titrated, were diluted in medium so that 0·1 ml. contained about 10^5 colony-forming units (c.f.u.). This was spread on the agar medium which had been dried for 45 min. at 37° before inoculation. Filter paper discs of 6 mm. diameter were soaked with 0·025 ml. of undiluted antiserum and were placed on the agar. The same technique was used with discs containing antibiotics. Zones of inhibition were measured from the edge of the disc to the edge of the area of colonies as soon as these were visible.

<center>RESULTS</center>

Identification of mycoplasma strains. The various strains of *Mycoplasma hyorhinis* were identified in preliminary gel-diffusion tests. Lines of precipitate formed between a well which contained a mycoplasma antigen and a well which contained its specific antiserum, and reactions of identity were seen with all strains tested against such an antiserum. All nine strains of *M. hyorhinis* were examined also by the m.i. technique, and there was a sufficiently close relationship between them to permit identification by this technique. The results of testing six strains against each of their respective antisera are shown in Table 1. The metabolism of all strains was inhibited by a single antiserum. Each of the antisera had a high antibody titre against its homologous organism, although an antiserum sometimes inhibited a heterologous strain only at a low titre. All other mycoplasma species antisera that were tested failed to inhibit the glucose metabolism of the F strain of *M. hyorhinis*, and an antiserum to this strain did not inhibit these other mycoplasma species (Taylor-Robinson & Dinter, 1968).

Table 1. *Relationship between six strains of Mycoplasma hyorhinis as shown by the metabolic-inhibition technique*

	Reciprocal of m.i. titre with rabbit antisera to					
Strain	F(1)*	S7	200	SK	G	GDL
F	**10240**	80	10240	640	5120	2560
S7	640	**640**	5120	10240	5120	5120
200	2560	2560	**20480**	5120	10240	5120
SK	160	160	1280	**2560**	160	160
G	2560	640	1280	1280	**1280**	2560
GDL	320	160	5120	10240	1280	**10240**

* F(1): antiserum to strain F from rabbit No. 1.

Sensitivity of Mycoplasma hyorhinis strains to antisera. Six strains and their respective antisera were tested comparatively by metabolic and disc growth-inhibition. In m.i. tests, the metabolism of each strain was inhibited by each antiserum, that against strain S7 having the weakest activity (Table 1). However, in disc growth-inhibition tests the growth of all strains was not inhibited (Table 2); two strains were inhibited by five antisera, two strains by three antisera and two strains were not inhibited by any of the antisera. Also, antiserum to strain S7 did not inhibit the growth of any of the strains. These results were thought to reflect differences in the strengths of antisera and in the sensitivity of particular strains to antibodies, especially when tested by the disc growth-inhibition method.

To examine more fully the sensitivity of the strains to antisera in the discs, growth-

inhibition tests were made with all the nine mycoplasma strains and their antisera. The results are summarized in Table 3; these show that antisera to strains 200, SK and GDL were strongest in that they inhibited the growth of more strains than did the other six antisera. Further, the growth of strain K was inhibited by all antisera, including the weakest antiserum (that to strain S7). The K strain was thus regarded as being the most 'sensitive'. Within the group of sensitive strains, strain GDL appeared to be least sensitive. The growth of four strains (G, W, S7, F/D) was not inhibited by any antiserum and these were regarded as being 'insensitive' strains.

Table 2. *Relationship between six strains of Mycoplasma hyorhinis as shown by the disc growth-inhibition technique*

	Zones of inhibition (mm.)* with rabbit antisera to					
Strain	F(I)†	S7	200	SK	G	GDL
F	3·7	0	3·7	2·7	2·7	2·5
S7	0	0	0	0	0	0
200	3·5	0	4·5	3·5	3·2	3·5
SK	0	0	3	4·5	0	3
G	0	0	0	0	0	0
GDL	0	0	2·5	3	0	3·5

* Zones < 1 mm. are indicated with o.   † F(I): antiserum to strain F from rabbit No. I.

Table 3. *A survey of strains of Mycoplasma hyorhinis sensitive and insensitive to antiserum in disc growth-inhibition tests*

	Presence (+)* or absence (−) of zones of inhibition with rabbit antisera to								
Strain	K	F(I)†	F(2)†	200	SK	GDL	G	W	S7
K	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	−
200	+	+	+	+	+	+	+	−	−
SK	−	−	+	+	+	+	−	−	−
GDL	−	−	−	+	+	+	−	−	−
G	−	−	−	−	−	−	−	−	−
W	−	−	−	−	−	−	−	−	−
S7	−	−	−	−	−	−	−	−	−
F/D	−	−	−	−	−	−	−	−	−

* Zone of inhibition ⩾ 2 mm.
† F(I) and F(2): antisera to strain F from rabbits No. I and 2, respectively.

Variations in the results of disc growth-inhibition tests. A liquid culture of the antiserum-sensitive strain F was mixed with various dilutions of a culture containing organisms (F/D or SK) insensitive to the antiserum used. The mixtures were then inoculated on agar medium and tested by disc growth-inhibition. The results are shown in Table 4. Variation in the proportion of antiserum-sensitive to insensitive organisms resulted in either the production of a zone of growth inhibition or the absence of such a zone. A similar variation in antiserum sensitivity was observed to occur on continued passage of a strain. Thus, the F/D strain emerged as an antiserum-resistant derivative after more than 12 subcultures of the sensitive F strain in liquid medium. Either this or variation in the proportion of antiserum-sensitive to resistant organisms in a liquid culture might explain why the results of disc growth-inhibition tests with

some of the strains were different from those observed previously (Dinter *et al.* 1965). Since that report, these strains have been subcultured several times in liquid media.

Since the growth of some mycoplasma strains was not inhibited by antiserum in discs on solid medium, but was inhibited by the same antiserum in liquid medium, it was thought that decrease in the concentration of agar might result in the formation of a zone of growth inhibition. However, with strain s7 no zone was formed on solid medium containing as little as 0·1 % agar. Further, the size of zones produced by antiserum in tests with strain F was not significantly increased by using this low concentration of agar. The inhibition of mycoplasma growth by antiserum in a disc was not changed by the substitution of agarose for agar, the addition of 1 % glucose to the agar, the use of unheated antiserum, or the addition of fresh guinea-pig serum to the antiserum.

Table 4. *Zone development with mixtures of* Mycoplasma hyorhinis *organisms sensitive and insensitive to antiserum*

No. of c.f.u.* of indicated organism in mixture of

'Antiserum sensitive' (F, passage 12)	+	'Antiserum insensitive' (F, passage 15)†	(SK)‡	Zone in mm. with antiserum F(1)
10^4		10^5	—	0
10^4		10^4	—	2·0§
10^4		10^3	—	2·4§
10^4		10^2	—	2·7
10^4		—	10^6	0
10^4		—	10^4	2·5§
10^4		—	10^3	3·6

* c.f.u.: colony-forming units.
† Later designated F/D.
‡ Insensitive to antiserum F(1) (see Table 3).
§ Colonies within the zone of general inhibition.

Formation of precipitation rings. Disc growth-inhibition tests were incubated up to 10 days after inoculation. Within this time, in some of the tests, rings of precipitate occurred in the agar medium around the discs containing antisera (Pl. 1, fig. 1). The antisera had been absorbed with concentrated medium before use, and when they were tested by gel diffusion they did not produce precipitation lines with concentrated extracts of the agar medium. Thus, the rings were produced apparently by the interaction of antiserum with antigens released into the agar from organisms in the colonies. The total number of rings produced by each mycoplasma strain exposed to various antisera, and by each antiserum exposed to various strains, was counted. The largest number of rings (8 to 11) was formed by antisera to strains 200, SK and GDL, and the smallest number (1 to 3) by antisera to the w and s7 strains. Within the group of 'antiserum sensitive' strains, the largest number of rings (up to 13) was produced by the K strain, and the smallest number (4) by the GDL strain. A small number of rings (1 to 4), often poorly developed, was produced by 'antiserum insensitive' strains. Thus, a rough correlation was apparent between the number of precipitation rings, the strength of antisera and susceptibility of the strains to antibodies.

Sensitivity of strains to polymyxins and other antibiotics. The occurrence of a greater

number of precipitation rings with antiserum-sensitive mycoplasma strains suggested that the limiting membrane of these strains was more susceptible to damage than was the membrane of antiserum-insensitive strains. To study this further, all the myco-plasma strains were tested against various antibiotics, some of which were known to inhibit the growth of bacteria by damaging their membranes. In preliminary tests commercial paper discs were used which contained the various antibiotics listed in Methods. Apart from the exceptions noted below, the growth of all the strains was inhibited by the antibiotics in the doses tested. Bacitracin and streptomycin did not inhibit the growth of any of the strains.

In preliminary tests with commercial paper discs the zones of growth inhibition produced by colistin sodium methanesulphonate (colistin: a polymyxin type) were greater in diameter when the agar medium was at pH 7·8 than when it was at pH 7·2, and in all subsequent tests the media were adjusted to pH 7·8. The results of the preliminary tests showed also that the antiserum-sensitive strains were also sensitive to polymyxins, while the antiserum-insensitive strains were resistant (Pl. 1, fig. 2).

Quantitative tests were then made in which various concentrations of colistin and

Table 5. *Inhibition of growth of strains of Mycoplasma hyorhinis by various amounts of colistin sodium methanesulphonate in discs*

| | Zones of inhibition (mm.) with colistin (μg./disc) | | | | | | Inhibition |
Strain	0	36	72	144	288	Standard*	(mean)
K	0	3	4	6·5	10	3	5·3
F	0	2·5	3	6	8	3	4·5
200	0	3	5	7	n.t.	5	5
SK	0	3·5	5	9	15	5	7·5
GDL	0	2·5	4·5	6	10	4	5·4
F/D	0	1·5	1	3	6·5	0	2·4
S7	0	0	1·5	4†	4	1·5	2·2
G	0	0	0	2†	3·5	0	1·1
W	0	0	1·5	3†	6·5	0	2·2

  * Commercial discs (900 i.u./disc).
  † Some colonies within the zone of general inhibition.
  n.t. = not tested.

Table 6. *Inhibition of growth of strains of Mycoplasma hyorhinis by various amounts of polymyxin B in discs*

| | Zones of inhibition (mm.) with polymyxin (μg./disc) | | | | | | Inhibition |
Strain	0	25	50	100	200	Standard*	(mean)
K	0	2·5	5	8	9	4	5·7
F	0	2·5†	3	5	7	4	4·3
200	0	2†	3·5	6	7·5	3	4·4
SK	0	2·5	5	7	11	3	5·7
GDL	0	2·5	3·5	6·5	9	2·5	4·8
F/D	0	0	1·5	2	4·5	0	1·6
S7	0	0	1·5	2·5	4	1·5	1·9
G	0	0	0	0	0	2·5†	0·5
W	0	0	0	0	1·5	0	0·3

  * Commercial discs (50 μg./disc).
  † Some colonies within the zone of general inhibition.

polymyxin B were tested against all the nine mycoplasma strains. The results are shown in Tables 5 and 6. The mean size of zones of inhibition produced by different concentrations of colistin was a little greater than the mean size of zones produced by polymyxin B. Again, the size of zones with antiserum-sensitive strains was greater than with antiserum-insensitive strains. However, within the group of antiserum-sensitive or polymyxin-sensitive strains there was no close correlation between the sensitivity of a mycoplasma strain to antisera and the degree of its inhibition by polymyxins (compare Tables 5 and 6 with Table 3). The critical concentration of polymyxin which differentiated sensitive from insensitive strains was 72 μg. colistin/disc and 25 μg. polymyxin B/disc.

In m.i. tests 100 μg. colistin/ml. decreased the number of colour-changing units (c.c.u./ml.) of all the mycoplasma strains by the same amount, and 250 μg. colistin/ml. completely or almost completely inhibited the metabolism of all the strains. Thus, the inhibition tests in liquid medium did not reveal differences between polymyxin-sensitive and polymyxin-insensitive strains.

Table 7. *Concentration of horse serum in agar medium required for the growth of strains of Mycoplasma hyorhinis*

	Growth of strains* on agar medium containing indicated concentrations (% v/v) of horse serum					
Strain	0	2	4	5	10	20
F	.	.	.	< 2	3	> 3
SK	.	.	.	< 2	3	> 3
200	.	.	< 2	2	3	> 3
K	.	.	< 2	2	> 3	.
GDL	.	< 2	3	> 3	.	.
F/D	< 2	2	3	> 3	.	.
S7	< 2	3	> 3	.	.	.
G	< 2	3	> 3	.	.	.
W	< 2	3	> 3	.	.	.

* < 2, 2 or 3, > 3 = respectively, no colonies, few colonies, many colonies developing after inoculation of agar medium with liquid cultures diluted 100-fold (2) or 1000-fold (3).

Influence of horse serum concentration on mycoplasmal growth. During these studies it was noted that some of the mycoplasma strains were able to grow on agar medium containing less than 20 % (v/v) horse serum. To investigate this, each strain was inoculated on agar medium which contained various concentrations of horse serum. Colonies of antiserum-insensitive strains developed on agar medium containing low concentrations of horse serum, while colonies of sensitive strains developed only on agar with higher concentrations (Table 7). However, within the group of antiserum-sensitive strains there was no correlation between the sensitivity to antisera and the degree of dependence upon horse serum for growth, although the GDL strain seemed to be least dependent (compare Table 7 with Table 3).

When the various mycoplasma strains were grown in liquid medium containing 5 % (v/v) horse serum, there was no difference in the amount of growth, in terms of colony-forming units, as between antiserum-sensitive and antiserum-insensitive strains. Furthermore, addition of various amounts of colistin to such a medium did not elicit detectable differences between the strains.

Influence of pH value of agar medium on mycoplasmal growth. Eight of the mycoplasma strains were each inoculated on agar medium at pH 7·8 and at pH 6·5. The number of colonies was counted after incubation for 4 days at 37°; the results are shown in Table 8. In general, the number of colonies of antiserum-sensitive strains which developed on agar medium at pH 6·5 was less than the number of colonies of antiserum insensitive strains. The mycoplasma strains were inoculated also into liquid medium at the two pH values. Samples were removed after various times of incubation at 37° and inoculated on solid medium. As judged by colony counts, all strains multiplied less in liquid medium at pH 6·5 than at pH 7·8. However, the growth (c.f.u./ml.) of antiserum-sensitive or antiserum-insensitive strains at pH 6·5 was the same.

Table 8. *Inhibition of development of colonies of Mycoplasma hyorhinis*
strains on agar medium at pH 6·5

Strain	Decrease $(\log_{10})$ in number of colonies*
K	> 3
F	> 3
200	> 4
SK	4
GDL	> 2
G	2
S7	Nil
F/D	Nil

* Compared with no. on agar medium at pH 7·8.

Size of mycoplasma organisms. The possibility that antiserum-sensitive strains consisted of organisms of a different size from antiserum-insensitive strains was considered. Liquid cultures of strains F and S7 were filtered through Millipore filters of different pore sizes; the proportion of organisms of each strain which passed through each filter was the same. Thus, there was no evidence to suggest that there was a difference in the size of organisms of the different mycoplasma strains.

DISCUSSION

The differences in the sensitivity of the nine *Mycoplasma hyorhinis* strains to specific antisera, polymyxins or pH 6·5 were observed only when they were grown on solid medium. It was not possible to differentiate the strains into sensitive and insensitive ones when the organisms were grown in liquid medium. Furthermore, growth of sensitive strains, as defined above, on solid medium required higher concentrations of horse serum than did the growth of insensitive strains. Such a difference was not seen when the various strains were grown in liquid medium.

The concept of sensitivity as used in the present paper is empirical. Those strains that were inhibited by all the antisera were regarded as most sensitive, those inhibited by fewer antisera as less sensitive and those not inhibited by any antisera as antiserum resistant. It is not known whether individual organisms are either sensitive or resistant and remain so on repeated passage, in which case the sensitivity of a particular mycoplasma strain would be dependent upon the proportions of the different organisms in

the culture, or whether sensitive organisms changed to insensitive, and vice versa. It is also difficult to explain why the various phenomena were observed only in tests on solid medium. Possibly strains that appeared insensitive to antiserum or polymyxins on solid medium were sensitive in liquid medium because of the opportunity for more intimate contact with antibody or antibiotic. Whatever the reason for the phenomena occurring on solid medium only, it seems clear that the differences between sensitive and insensitive strains, whether the sensitivity is to antiserum or polymyxins, may be related to the mycoplasma membrane. There is evidence that growth-inhibitory antisera are directed against antigens in or on the mycoplasma membrane (Williams & Taylor-Robinson, 1967). The mechanism of mycoplasmal growth-inhibition by polymyxins is unknown, but it is known that polymyxins inhibit the growth of bacteria by damaging their membranes (Newton, 1956; Sebek, 1967). Well-defined precipitation rings occurred in disc growth-inhibition tests in which *Mycoplasma hyorhinis* growth was inhibited by antiserum. Such precipitation is not confined to tests with *M. hyorhinis* since, occasionally, similar precipitation rings have been observed in studies of other mycoplasma species. In the case of *M. hyorhinis* it seems that the phenomenon is indicative of membrane damage with lysis of the organisms. Lysis with liberation of antigens must have occurred, since in tests with antiserum-insensitive strains, although antigen was potentially available because of profuse colony growth, rings of precipitation were few in number and were poorly developed. Thus, the theory may be put forward that specific antibodies attach to the mycoplasma membrane, damage it and cause leakage of specific antigens into the agar, and that the membranes of sensitive organisms are more susceptible to damage by antibodies in this way than those of insensitive strains.

Newton (1956) analysed cell walls from a polymyxin-sensitive and a polymyxin-resistant strain of *Pseudomonas aeruginosa*. A difference in the phospholipid content of the two strains was thought to be a factor contributing to the selective action of polymyxin, the phospholipid content being greater in the sensitive than in the resistant strain. The dependence for growth of some of our antiserum-sensitive mycoplasma strains on high concentrations of horse serum does not appear to contradict this. Horse serum supplements the medium with protein, cholesterol and phospholipid, known to be required for the reproduction of parasitic mycoplasma strains (Razin, Cosenza & Tourtellotte, 1967), and which are incorporated into the mycoplasma membrane. It has been shown by Sobeslavsky, Prescott, James & Chanock (1966) that lipid in the membrane of *Mycoplasma pneumoniae* is concerned in growth inhibition by antiserum. These authors were able to block the effect of growth-inhibitory antiserum with lipid extracted from this organism. Possibly quantitative changes in the phospholipid composition of *M. hyorhinis* organisms could account for their changes in antiserum sensitivity. A change in the antiserum sensitivity of a strain on serial passage might be due to variation in the composition of the mycoplasma membrane. The fact that the growth of antiserum-sensitive strains in the present experiments was suppressed on agar medium at pH 6·5 may be attributable also to the phospholipid content of their membranes. It has been shown for some bacteria (van Deenan, 1965) that a change in their phospholipid distribution could be attributed to a decrease of the pH value of the medium. Such could be the case with mycoplasmas.

Hayflick & Stanbridge (1967) studied a strain of *Mycoplasma hyorhinis* and considered that it contained a population of organisms, some sensitive and others resistant

to antiserum. They found colonies produced by apparently resistant organisms in the zone of growth inhibition produced by antiserum-sensitive organisms. We have not observed this particular phenomenon to occur naturally although it has been possible to reproduce it experimentally with mixtures of sensitive and insensitive strains (Table 4). However, our findings are consistent with the concept of antiserum-sensitive and resistant organisms, and it seems reasonable to postulate that these phenomena are attributable to changes in the composition of the mycoplasma membranes.

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EXPLANATION OF PLATE

Fig. 1. Disc growth-inhibition tests with the K strain of *Mycoplasma hyorhinis*. Zones of inhibition and rings of precipitation with, from left to right, rabbit antisera SK, F and 200. Day 9 after inoculation.

Fig. 2. Disc growth-inhibition tests with the sensitive K strain (above) and the insensitive F/D strain (below) of *Mycoplasma hyorhinis*. Discs on left contained rabbit antiserum 200 (undiluted); discs on right contained colistin (900 i.u.). Day 4 after inoculation.
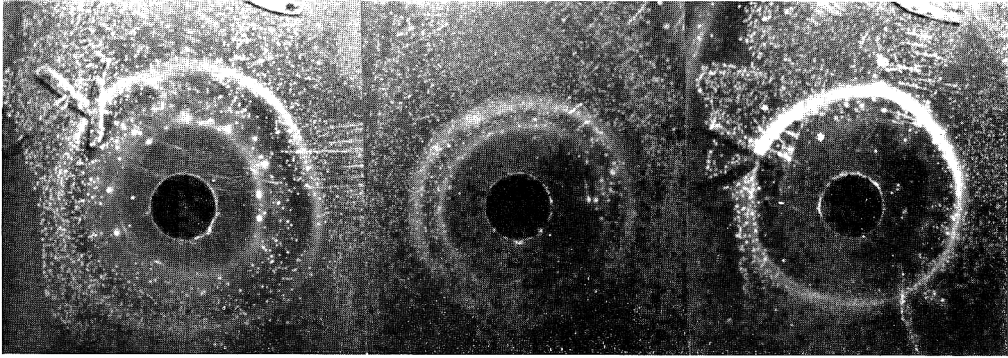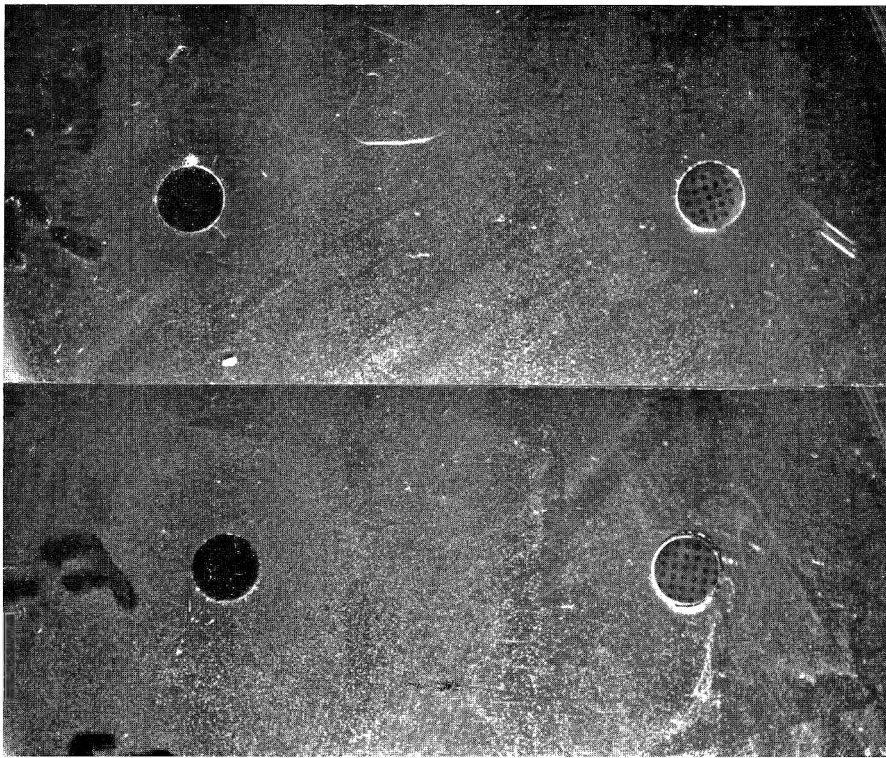
Fig. 1

Fig. 2

Z. DINTER AND D. TAYLOR-ROBINSON

Butyramide-utilizing Mutants
of *Pseudomonas aeruginosa* 8602 which Produce an Amidase
with Altered Substrate Specificity

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SUMMARY

Mutants of *Pseudomonas aeruginosa* 8602 were isolated which, unlike the wild type, were able to grow with butyramide as a carbon source. Six mutants derived from the constitutive strain C 11 were shown to produce an enzyme (B amidase) with altered electrophoretic mobility and altered substrate specificity. The apparent K_m for butyramide of the B amidase was about a tenth of that of the A amidase and the V_{max} was about ten-fold greater. A further mutation produced mutants able to grow on valeramide.

INTRODUCTION

Pseudomonas aeruginosa 8602 grows in a minimal medium containing either acetamide or propionamide as a carbon + nitrogen source, and both these amides induce the synthesis of an aliphatic amidase (acylamide amidohydrolase EC 3.4.14). This organism is unable to utilize butyramide for growth and Kelly & Clarke (1962) found that acetamide-grown bacteria (and extracts prepared from them) had very little amidase activity with butyramide as a substrate and that butyramide did not induce amidase synthesis in a succinate + minimal salt medium. The magno-constitutive mutant C 11 (Brammar, Clarke & Skinner, 1967) is also unable to grow on butyramide and produces an enzyme which is indistinguishable from the wild-type amidase. We have now obtained mutants of strain C 11 which grow on agar plates and in liquid medium with butyramide as the carbon source. The amidase produced by one of these butyramide-utilizing mutants has been purified and compared with that produced by strain C 11.

METHODS

Organisms. The parent strain was *Pseudomonas aeruginosa* 8602. Strain C 11 was a spontaneous mutant, constitutive for amidase (Brammar *et al.* 1967) and was used to isolate the butyramide-utilizing mutants designated B 1 to B 6 (see later for genetic descriptions).

Media. The minimal salt medium described by Brammar & Clarke (1964) was used for all growth experiments; solid media contained 1·2 % (w/v) Oxoid No. 3 agar. Lactate and pyruvate media were prepared by adding sterile solutions of the sodium or potassium salts aseptically to autoclaved minimal salt medium. Butyramide plates contained 0·1 % (w/v) and valeramide plates 0·2 % (w/v) of the specific amide, and were prepared by adding sterile solutions of the amides to minimal agar. Other media were prepared as described by Brammar *et al.* (1967).

Mutagen treatment. Bacteria were grown overnight with shaking at 37° in 5 ml. of nutrient broth. The bacteria were centrifuged down, resuspended in 4 ml. citrate buffer (0·1 M; pH 6·0), 1 ml. aqueous solution of N-methyl-N'-nitro-N-nitrosoguanidine (1 mg./ml.) was added and the suspension allowed to stand for 40 min. at room temperature. The bacterial suspension was centrifuged again, the deposit suspended in 5 ml. dilution buffer and 0·2 ml. of this spread on each of several butyramide or valeramide plates.

Transduction. Phage lysate preparation and transduction procedure were as described by Brammar *et al.* (1967).

Growth of bacteria for enzyme studies. Organisms for enzyme experiments were grown at 37° in conical flasks of capacity 5 to 10 times the volume of medium and were shaken. For enzyme preparations the bacteria were grown in 5 l. conical flasks containing 1 l. of 0·5 % (w/v) pyruvate or lactate medium.

Preparation of cell-free extracts. Bacteria grown overnight in 200 ml. of 1 % (w/v) pyruvate medium were centrifuged down and then suspended in 2 ml. of ice-cold tris buffer (0·1 M; pH 7·2). The bacteria were disrupted by ultrasonic treatment for 2 min. at 0° by using an MSE 25 W ultrasonic oscillator. Debris was removed by centrifugation at 25,000 g for 20 min. at 4°. The supernatant fluids were stored at $-30°$. Large scale preparations of extracts for enzyme purification were made by re-suspending bacteria (equiv. 65 mg. dry wt/ml.) in ice-cold tris buffer and disrupting in a Ribi-Sorvall fractionator at a pressure of 15,000 to 20,000 p.s.i. (lbs/inch²); the needle valve was cooled throughout the operation by a stream of nitrogen at 0°.

Enzyme purification. The mixture obtained after cell disruption was treated with streptomycin sulphate (1 g./6 g. protein), homogenized and centrifuged at 11,000 g for 30 min. at 4° to remove cell debris and precipitated nucleic acid; 250 ml. portions of the supernatant fluid were heated rapidly to 60°, maintained at that temperature for 8 min. and then cooled rapidly to 0°. The heat-treated solutions were bulked and centrifuged at 11,000 g for 20 min. at 4°. Fractionation of the supernatant fluid was done by ammonium sulphate precipitation; precipitates were collected by centrifugation (11,000 g for 20 min.) after 50, 55, 65 and 75 % saturation with ammonium sulphate. At each stage the solutions were allowed to stand at 0° for 30 min. before centrifuging. The P_{65} fraction (55 to 65 % saturation with ammonium sulphate) contained the bulk of the amidase activity and was dissolved in tris (0·1 M; pH 7·2) buffer containing 0·15 M-KCl, dialysed against two changes of the same buffer and centrifuged at 15,000 g to remove any precipitate. The dialysis and subsequent operations were done at room temperature. The protein solution was loaded on to a column of DEAE-Sephadex previously equilibrated with the same buffer; the column was washed for 2 hr with this buffer before a linear concentration gradient from 0·15 M to 0·35 M-KCl in a total volume of 2 l. tris buffer was applied to the column. Fractions containing the major 280 mμ-absorbing peak were assayed for amidase and samples from each fraction examined by disc electrophoresis in polyacrylamide gels (Davis, 1964). Fractions shown to be homogeneous for the amidase enzyme by this method were bulked. A small portion of this material was retained for enzyme work and stored at $-30°$; the bulk was dialysed against 0·1 M-KCl and precipitated by exhaustive dialysis against deionized water. The precipitate was extracted with ethanol+ether and stored at 4° for use in structural studies.

Enzyme assays. Enzyme activity was routinely measured by the transferase assay

described by Brammar & Clarke (1964). Final concentrations of amides in the substrate mixture were acetamide 0·1 M, propionamide 0·125 M, butyramide and iso-butyramide 0·15 M. Hydrolase activity was measured by estimating the ammonia produced in the reaction by the Conway microdiffusion method (Kelly & Clarke, 1962). This method could not be used with valeramide, since in the presence of saturated K_2CO_3 valeramide formed a film on the surface of the reaction mixture which prevented ammonia diffusion. With valeramide as a substrate in the hydrolase reaction the ammonia was measured by its reaction with ninhydrin. Samples (0·1 ml.) were removed from the incubation mixture (bacterial suspension; 0·1 M, pH 7·2 tris buffer; 0·2 M-valeramide) and added to 1 ml. 0·2 M-citrate buffer (pH 5·0), 1 ml. ninhydrin reagent added, the mixture heated at 100° for 15 min. and after the addition of 5 ml. 50 % (v/v) ethanol in water the extinction was measured at 570 mμ with a Unicam SP 600 spectrophotometer. The ninhydrin reagent (modification suggested by P. D. Laverack) was freshly prepared 1 hr before use by adding 0·1 ml. KCN solution (0·1 M-KCN diluted 1/15) dropwise to 10 ml. 2-methoxyethanol followed by 2 ml. 3 % (w/v) ninhydrin in 2-methoxyethanol. Ester hydrolase and transferase activities were measured by the methods described by McFarlane, Brammar & Clarke (1965).

Enzyme units. One unit of transferase activity is defined as the amount of enzyme catalysing the formation of 1 μmole acylhydroxamate/min. under the standard assay conditions. One unit of hydrolase activity is defined as the amount of enzyme cata-lyzing the formation of 1μmole ammonia/min. under the standard assay conditions.

Estimation of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for protein estimation, with bovine plasma albumin as a standard.

Buffers. Tris buffer, 0·1 M, pH 7·2, was used to prepare bacterial suspensions and for enzyme assays; 0·001 M-EDTA and 0·025 % (v/v) mercaptoethanol were added for assays with purified enzyme preparations and at all stages of amidase purification. Barbitone + acetate buffer, pH 8·6, ionic strength I 0·05, +0·01 M-NaN_3 was used for immunodiffusion tests.

Reagents. Tris (2-amino-2-hydroxymethylpropane-1:3 diol) was obtained as 'Trizma base' from the Sigma Chemical Co. Acetamide obtained from Hopkins & Williams Ltd. was recrystallized twice from ethanol before use. Propionamide and butyramide were obtained from British Drug Houses Ltd. and were recrystallized twice from ethanol. Isobutyramide was prepared from isobutyryl chloride and ammonia and was recrystallized from ethyl acetate. Hydroxylamine hydrochloride from Koch-Light Ltd. was recrystallized twice from ethanol + water (3 + 1, v/v). N-methyl-N'-nitro-N-nitrosoguanidine was obtained from the Aldrich Chemical Co. Inc. Hydrolysed starch was obtained from Connaught Medical Research Laboratories. Esters were obtained from British Drug Houses Ltd. and were used without further purification. Complete Freund's Adjuvant was obtained from Difco Laboratories Inc. Deionized water was used to prepare all solutions involved in the enzyme purification.

Starch gel electrophoresis. Starch gel electrophoresis was done as described by Gammack, Huehns, Shooter & Gerald (1960) with the discontinuous tris + citrate + borate buffer system at pH 8·5. Gels were sliced and one slice was stained for protein with a saturated solution of naphthalene black in methanol + water + acetic acid (5 + 5 + 1 by vol., for 5 min.). The background stain was washed out with the same solvent. Gel slices were stained for amide hydrolase activity by overlaying with a filter paper soaked with a solution of the appropriate amide, incubating for 15 min. at 37°

and replacing the filter paper by one soaked in Nessler's reagent. Yellow bands on the filter paper indicated the position of the amidase protein. Amide transferase activity was detected by incubating a gel slice overlaid with a filter paper soaked in the mixed substrate solution for 15 min. at 37°, and replacing the filter paper by one soaked in the ferric chloride reagent; the position of the amidase was shown by the formation of a brown band on the surface of the gel.

Immunodiffusion. Rabbit antiserum against the purified C11 enzyme was prepared by giving rabbits two intramuscular injections, spaced by an interval of 4 weeks, of 0·75 ml. of an emulsion of equal volumes of a solution of purified enzyme (8 mg./ml. in tris buffer) and Freund's complete adjuvant. An intravenous booster dose of 0·5 ml. of enzyme solution was given after a further 2 weeks. The rabbits were bled a week later; blood was allowed to clot at 4°, centrifuged and the serum obtained was stored at −30°. The serum was not further purified and was used either undiluted or diluted 1/2 with normal saline in immunodiffusion tests. Immunodiffusion was done in agar gels (2 ml. 1 % (w/v), Difco agar Noble in barbitone + acetate buffer on microscope slides, 2·5 × 7·5 cm.). Wells were cut with a No. 2 cork borer; 10 μl. samples were placed in the outer wells and 10 μl. antiserum in the centre well. Precipitin lines were developed in a moist environment at room temperature, or in the case of the extracts of V mutants at 4°, and were photographed after 20 hr.

RESULTS

Isolation of butyramide-utilizing mutants

Pseudomonas aeruginosa strain C11 is a spontaneous constitutive mutant which was isolated on succinate + formamide (SF) plates (Brammar *et al.* 1967). Formamide can be hydrolysed by induced bacteria at about one-sixth the rate for acetamide but formamide has almost no amidase-inducing activity for the wild-type strain. Constitutive mutants have therefore a selective advantage over the wild type in their ability to use formamide for growth. *P. aeruginosa* cannot metabolize C_1 compounds so that formamide provides only the nitrogen source and succinate is added as carbon source. It was argued that a single mutation in the amidase structural gene of strain C11, which produces a very high amidase activity in the absence of inducer, might produce a new mutant which would be able to grow on butyramide. After mutagenic treatment, about 10^7 bacteria were plated on butyramide agar and after 2 to 3 days colonies appeared. A few of the larger ones were picked off and re-isolated from butyramide agar. The mutants chosen for further study were designated B1 to B6. The growth characteristics of the mutants and the parent strains are given in Table 1. Brammar *et al.* (1967) showed that for a series of regulator mutants, all of which produced wild-type enzyme, the character for constitutivity was co-transduced with the amidase-positive character and concluded that an amidase regulator gene was closely linked to the amidase structural gene. In previous investigations the amidase mutants have been named so as to indicate their phenotypic behaviour but we now propose to introduce the genetic descriptions *amiR* for the amidase regulator gene and *amiE* for the amidase structural gene. This follows the recommendations of Demerec, Adelberg, Clark & Hartman (1966).

We have previously isolated a number of amidase-negative mutants (Skinner & Clarke, 1968) and these have now been assigned the genetic descriptions *amiE1* to

amiE10. The genetic description of the regulator gene mutants, including both constitutive and formamide-inducible mutants (Brammar *et al.* 1967), becomes *amiR1* to *amiR30*. It is particularly convenient for the other investigations we are doing to retain a very simplified nomenclature indicating the phenotype, and for general descriptive purposes we are continuing to use our previous nomenclature, e.g. C11 indicates a constitutive strain isolated on succinate+formamide agar, producing wild-type enzyme and with a mutation (*amiR11*) in the amidase regulator gene. The butyramide mutants described in the present paper were isolated from strain C11 and shown to produce altered enzymes. Mutant B6 is therefore a constitutive mutant which produces a mutant enzyme protein and has the genetic description *amiR11amiE16* (Table 1).

Table 1. *Growth characteristics of Pseudomonas aeruginosa 8602*
wild type and mutants

Strain...	Wild type	C11	B16
Genotype	*amiR+amiE+*	*amiR11amiE+*	*amiR11amiE16*
Phenotype	Inducible	Constitutive	Constitutive
Amidase	A enzyme	A enzyme	B enzyme
Growth on minimal agar plates			
+Acetamide	+	+	+
+Succinate/Formamide	−	+	+
+Butyramide	−	−	+

+ = Growth on plates after 3 days incubation at 37°; − = no growth.

Amidase activities of intact bacteria

Kelly & Clarke (1962) showed that for the wild-type *Pseudomonas aeruginosa* 8602 strain, although acetamide was the most potent of the substrate inducers, the amide most rapidly hydrolysed was propionamide. The enzyme is also able to transfer the acyl moiety of the amides to hydroxylamine to form hydroxamates. It was found that for this reaction the rate of hydroxamate formation from acetamide was greater than that from propionamide. During growth the physiological role of the enzyme is amide hydrolysis so that it is reasonable to expect a difference in the rates of hydrolysis of butyramide as between mutants able to grow on butyramide and the parent strain C11 which is unable to do so.

Figure 1 compares the rates of hydrolysis of formamide, acetamide, propionamide, lactamide, isobutyramide and butyramide by washed suspensions of strain C11 and its mutant B6. The hydrolysis rates for the various amides are expressed in relation to an arbitrary value of 100 for acetamide hydrolysis. The specific acetamide hydrolase activities of these two strains grown under the same conditions did not differ markedly, although it appeared that the cultures of the B mutants were usually slightly less active than those of strain C11. It can be seen that the relative rate of butyramide hydrolysis by mutant B6 was about 15 times that of C11 and that B6 also had a higher relative rate for the hydrolysis of propionamide, lactamide and isobutyramide. The substrate profiles for hydrolase activity for mutants B1 and B2 did not differ significantly from that obtained for B6.

Figure 2 compares the relative transferase activities of strains C11 and B1 and shows that B1 was much more active than C11 with the longer chain amides as substrates.

Only trace activity (< 1%) was detected with butyramide and isobutyramide as transferase substrates for strain C11, but the relative transferase activities of mutant B1 with these two substrates were 12 and 3 %, respectively, of the rates with acetamide. The relative rate of propionyl transfer by B1 was twice that of C11. The substrate profiles for transferase activity of the other five mutants (B2 to B6) were, as for the hydrolase activities, not significantly different from that shown for mutant B1.
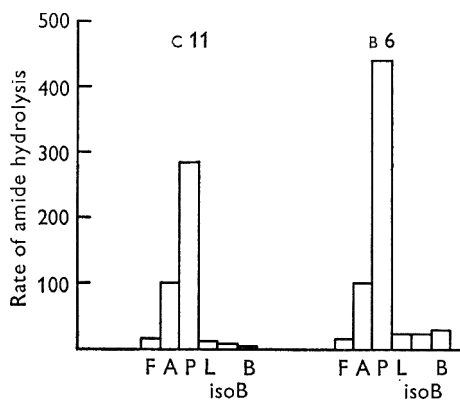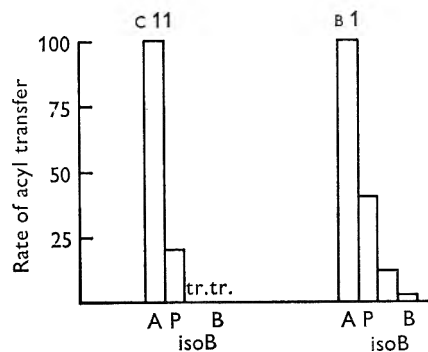

Fig. 1   Fig. 2

Fig. 1. Relative hydrolase activities of washed suspensions of *Pseudomonas aeruginosa* 8602 strain C11, and strain B6. F, formamide; A, acetamide; P, propionamide; L, lactamide; isoB, isobutyramide; B, butyramide.

Fig. 2. Relative acyl transferase activities of washed suspensions of *Pseudomonas aeruginosa* 8602 strain C11, and strain B1. A, acetamide; P, propionamide; isoB, isobutyramide; B, butyramide; tr, trace.

The increased amidase activities of the B mutants on the higher molecular-weight amides might have been due to differences in amide permeability. Brammar, McFarlane & Clarke (1966) showed that the wild type possesses a constitutive permease which enables it to concentrate both acetamide and N-acetylacetamide from the medium. Although the permeability of the bacteria for butyramide and the other amides has not been tested directly it seems unlikely that the difference in amidase activities between strain C11 and the B mutants could be accounted for in terms of permeability. *Pseudomonas aeruginosa* 8602 and its mutants appear to be freely permeable to amides (perhaps *via* a single permease) and the relative acyl transferase activities of C11 and the B mutants were found to be the same with cell extracts as with whole bacteria.

Amidase activity of bacterial extracts

Magno-constitutive mutants grown in pyruvate or lactate media, in which there is little catabolite repression, produced large amounts of amidase constituting 5 % or more of the total bacterial protein. When cell-free extracts were subjected to starch-gel electrophoresis, the amidase was observed as a major protein band. One of the slices of the gel was stained with naphthalene black for protein and other slices were stained for acetamide hydrolase and transferase activities. The amidase band in extracts of strain B6 moved more slowly than that of strain C11. In mixtures of extracts from the two strains the two separate amidase bands could be identified. The amidase band

from extracts of B6 and the slower moving band in the mixtures gave strong butyr-amide hydrolase and transferase reactions. Slight butyramide hydrolase activity was shown with C11 extracts when the gels were very heavily loaded and this was always associated with the acetamide hydrolase and transferase activities. These observations suggested that the differences between strain B6 and strain C11 were due to the production of an amidase with altered substrate activity. The electrophoretic mobilities of the amidases in cell extracts of all the B mutants were identical.

Comparison of C11 and B6 amidases

From the preceding observations it was concluded that mutant B6 was able to grow on butyramide because it produced an altered enzyme (B enzyme) as a result of a mutation in the gene which determines the structure of the wild-type amidase (A enzyme). This relationship was confirmed by genetic studies. In transduction tests, with B mutants as donors and amidase-negative mutants as recipients, amidase-positive transductants were found at a frequency comparable with that obtained in crosses between the wild type and amidase-negative mutants. Approximately 90 % of the amidase-positive transductants in the crosses with the B mutants were shown by replication tests to be also butyramide-positive and constitutive. This association of constitutivity with the amide-positive character was similar to that found previously in crosses between constitutive amidase-positive mutants and inducible amidase-negative mutants (Brammar *et al.* 1967).

Table 2. *Purification and recovery of A amidase from*
Pseudomonas aeruginosa strain C11

Amidase A from strain C11 grown in 100 l. minimal salt medium containing 1 % (w/v) sodium lactate was purified 19-fold. Amidase activity was determined as acetamide transferase. $(NH_4)_2SO_4$ fractions were resuspended in the minimal volume of tris buffer and dialysed before assay. Fraction P_{65} was loaded on a DEAE-Sephadex column.

	Volume (ml.)	Total enzyme units $\times 10^{-4}$	Total protein (g.)
Bacterial suspension	1100	730	c. 80
Cell-extract after strepto-mycin and heat treatment	1120	650	13·2
$(NH_4)_2SO_4$ fractions			
P_{50} (0–50 % sat.)	—	5·4	4·35
P_{55} (50–55 % sat.)	—	2·8	0·068
P_{65} (55–65 % sat.)	—	540	3·6
P_{75} (65–75 % sat.)	—	65	4·85
*+Tube 50 from DEAE-Sephadex column	3·7	8·8	0·051

* Specific activity of amidase in Tube 50 = 1700; +, total enzyme recovered from tubes around amidase peak 2×10^6 units $\equiv$ 30 % of amidase in cell-extract.

Table 2 shows the recovery of amidase activity at various stages of a typical purification procedure of a preparation from strain C11. The final step involved chromatography on DEAE-Sephadex; the elution profile for the C11 enzyme is shown in Fig. 3. The purification was monitored by disc electrophoresis on acrylamide gels. There was no significant difference in the behaviour of the A and B enzymes at any stage in purification. The ratio of acetamide to butyramide transferase activities of the B

enzyme remained constant throughout purification with a ratio of 11:1 with whole bacteria, 10·5:1 with cell extracts and 10:1 with the pure enzyme preparation.

Immunodiffusion experiments showed that there was complete cross-reaction between cell extracts, or purified amidase preparations, of strains C11 and B6 with antiserum prepared against the purified enzyme from strain C11. Complete cross-reaction was also found in the reciprocal experiment with antiserum prepared against the purified amidase from strain B6.
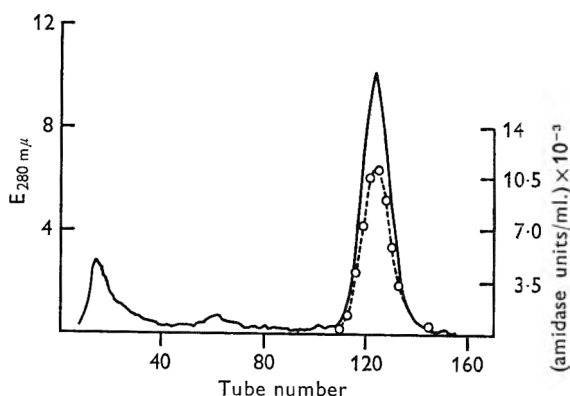

Fig. 3. Chromatography of amidase A from *Pseudomonas aeruginosa* 8602 strain C11 on DEAE-Sephadex. Elution of amidase by gradient of KCl in tris buffer (see Methods); column loaded with $(NH_4)_2SO_4$ fraction P_{65}. Amidase assayed by acetamide transferase reaction. - - - ○ - - - ○ - - -, amidase μmole acethydroxamate/min./ml.; ——, protein (extinction 280 mμ). 6·3 ml. fractions were collected.

Both A and B amidases were very stable in crude bacterial extracts; these could be stored at $-30°$ for several months without loss of activity. The purified preparations, even in the presence of protective agents such as mercaptoethanol and EDTA, slowly lost activity on prolonged storage. It was difficult to obtain very accurate values for the specific activity of the pure enzyme since some activity was always lost during the preparation of the very dilute solutions necessary for enzyme assays. The highest values for the A enzyme gave a specific activity for acetamide transferase of about 1800 μmole acethydroxamate/mg. protein/min. The highest value obtained for the B enzyme suggested that its acetamide transferase activity was about 80% of that of the A enzyme. The results with whole bacteria had suggested that the specific acetamide hydrolase and transferase activities/mg. dry wt bacteria of cultures of the B mutants were slightly lower than those of strain C11 grown in the same medium. Fig. 4 compares the hydrolase activities of purified preparations of A and B amidases on acetamide and butyramide. The rate of hydrolysis of butyramide by the A enzyme was only a small fraction (about 2%) of the rate of hydrolysis of acetamide, while the rate of hydrolysis of butyramide by the B enzyme was about 30% of the rate of acetamide hydrolysis. The specific butyramide hydrolase activity of the B enzyme was therefore about 10 times that of the A enzyme.

When determinations were made of the apparent Michaelis constants of the A and B amidases in the transferase reaction for acetamide, propionamide and butyramide, by using the Lineweaver–Burk method, it was evident that at very high butyramide concentrations the A enzyme had low but significant activity on butyramide (Fig. 5).

In all our subsequent estimations the concentration of butyramide in the substrate mixture for both hydrolase and transferase assays was increased to 0·5 M. Kelly & Kornberg (1964) had previously used 0·2 M-butyramide to assay purified amidase from the wild-type strain and at this concentration had not been able to detect any butyramide hydrolase or transferase activity.
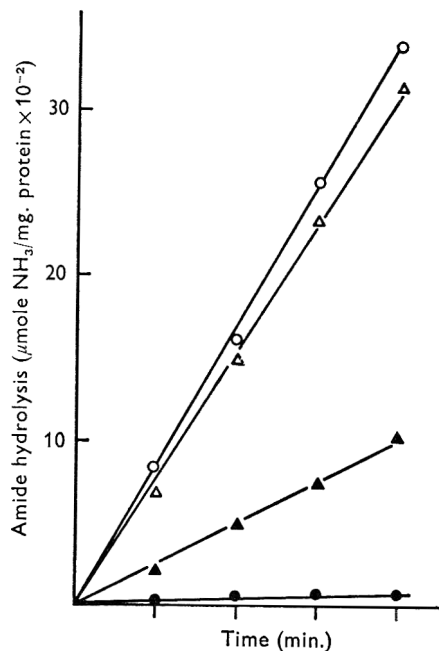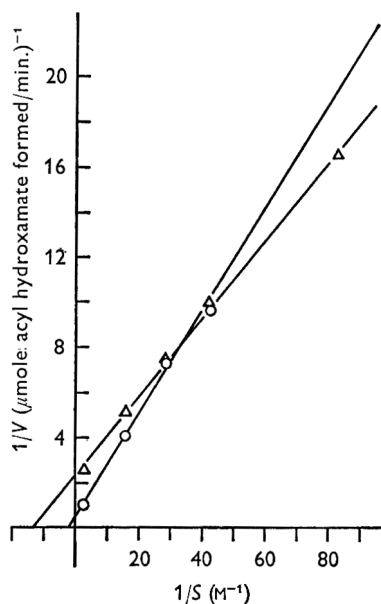

Fig. 4.

Fig. 5.

Fig. 4. Amide hydrolysis by A and B amidases. Enzyme activity measured by the Conway microdiffusion method. Amide concentration 0·2 M. A amidase on acetamide, ○ - - - ○; on butyramide, ● - - - ●. B amidase on acetamide, △ - - - △; on butyramide, ▲ - - - ▲.

Fig. 5. Lineweaver–Burk plot for determination of K_m for butyramide in the transferase reaction of A and B amidases. ○ - - - ○, A amidase; △ - - - △, B amidase.

Table 3. *Comparison of A and B amidases: apparent Michaelis constants*

	A amidase. *Apparent K_m mM	B amidase. *Apparent K_m mM
Acetamide	19	15
Propionamide	55	9·5
Butyramide	500	73·5

* Determined for the transferase reaction in the presence of excess hydroxylamine.

Figure 5 shows that there was about a ten-fold difference in the apparent K_m for butyramide of the A and B enzymes. The theoretical V_{max} values obtained from the same plot also showed about a 10-fold difference. The V_{max} of the A amidase for butyramide was 19·5 μmole hydroxamate/mg. protein/min. and the V_{max} for the B amidase for butyramide was 190 μmole hydroxamate/mg. protein/min. The apparent K_m of the B enzyme for propionamide was also less than that of the A enzyme

but there did not seem to be any significant difference in the K_m values of the two amidases for acetamide (Table 3).

Esterase activity of A and B amidases

McFarlane *et al.* (1965) showed that the wild-type amidase also had limited activity as an esterase. The rate of both hydrolase and transferase activity of the purified enzyme with ethyl acetate as substrate was about 1 % of that with acetamide in the same reactions. The esterase activity of the B enzyme was comparable to that of the A enzyme but ethyl propionate, isopropyl acetate and butyl acetate were more rapidly attacked (Table 4). These esters are all only very slightly soluble in water and all determinations were made with saturated aqueous solutions. This is probably well below substrate saturating concentration of the enzyme since McFarlane *et al.* (1965) found that the apparent K_m for ethyl acetate, the most water-soluble of the esters used, was 660 mM. However, it is clear that the differences in substrate specificity of the A and B amidases also extends to the ester substrates.

Table 4. *Ester transferase activity*

	A amidase		B amidase.
	Hydrolase	Transferase	Transferase
Methyl acetate	70	72	ND
Ethyl acetate	100	100	100
n-Propyl acetate	62	67	109
Isopropyl acetate	0	10	ND
Butyl acetate	ND	147	178
Ethyl formate	ND	65	ND
n-Propyl propionate	0	1·2	9·2
Isopropyl propionate	0	0	0

The esterase activity is expressed relative to that for ethyl acetate arbitrarily assigned a value of 100. ND = Not determined.

Table 5. *Valeramide-utilizing mutants*

Mutant	*Growth on amides			†Amide hydrolysis (μmole NH$_3$/mg. bact./min.)		
	Acet.	But.	Val.	Acet.	But.	Val.
V 1	+	+ + +	+ +	0·49	2·7	0·44
V 2	−	+ + +	+ +	ND	ND	ND
V 3	+ + +	+ + +	+ +	3·0	6·5	0 61
V 4	+ + +	+ + +	+ +	3·1	8·1	0·56
V 5	tr	+ + +	+ +	0·85	3·1	0·64
V 6	+ +	+ + +	+ +	3·7	9·3	0·78
V 7	+	+ + +	+ +	0·68	3·1	0·7
V 8	+ + +	+ + +	+ +	3·4	8·1	0·62
V 9	+	+	+ +	0·8	0·98	0·48
V 10	+	+	+ +	0·55	0·57	0·38
V 11	+	+	+ +	0·83	0·56	0·42
B 6	+ + +	+ + +	−	9·4	3·3	0
C 11	+ + +	−	−	15·5	0·3	0

* Growth was tested on minimal agar plates containing 0·5 % (w/v) acetamide, 0·1 % (w/v) butyramide acid or 0·2 % (w/v) valeramide and recorded after 3 days at 37°. + + +, + +, +, tr. = relative growth. − = no visible growth; † Amide hydrolysis was measured by the ninhydrin method. ND = Not determined.

Valeramide-utilizing mutants

One of the butyramide-utilizing mutants, B6, was used to isolate a further series of mutants able to grow on valeramide. These formed a heterogenous group and a few of them have lost the ability to grow on acetamide agar. Table 5 compares the hydrolase activity of whole bacteria of the v series of mutants with acetamide, butyramide or valeramide as substrates. For all these mutants the specific acetamide hydrolase activity was less than that of cultures of strains C11 and B6 grown under the same conditions. Several of the v mutants had much higher specific butyramide-hydrolase activities than strain B6 and washed suspensions of all the v strains were able to hydrolyse valeramide at a significant rate.

The amidase proteins of all the v mutants appeared to be much less stable than the A and B amidases since the enzyme activity was rapidly lost when the bacteria were disrupted. The extracts were examined by immunodiffusion against the antiserum to the A and B amidases and for all those tested the cross-reaction obtained was only partial. The double mutation in the amidase structural gene of the v mutants appears to have produced alterations in the enzyme protein which affect the antigenic specificity as well as the substrate specificity.

DISCUSSION

The double mutation which has changed the wild-type *Pseudomonas aeruginosa* 8602 into mutant B6 has increased its range of growth substrates. It may be considered as a positive evolutionary step for this organism in that it can now grow on a substrate which the wild type cannot use. The procedure of starting from a constitutive mutant seemed logical since butyramide appeared to have no inducing activity for the wild-type strain (Kelly & Clarke, 1962). However, this procedure was less straightforward than it at first appeared. Butyramide, like cyanoacetamide, represses the induction of amidase by N-acetylacetamide in the wild-type strain and it also represses to a considerable extent the synthesis of amidase by the constitutive strain C11 (Brown, 1969). The mutation which produced strain B6 from C11 affected the properties of the enzyme, but not its regulation, and the rate of amidase synthesis by strain B6 appears to be repressed by butyramide to about the same extent as the synthesis by C11. Mutant B6 is therefore able to grow in butyramide medium because the B amidase has a higher activity than the wild-type A amidase for butyramide as substrate. On the other hand, we have isolated other mutants which grow on butyramide but produce wild-type A amidase. These are constitutive mutants which have also become relatively insensitive to butyramide repression. In butyramide medium they synthesize large amounts of A amidase and, although the specific activity towards butyramide is much lower than that of the B amidase, the total amount of enzyme produced by the culture is sufficient to allow growth to occur (Brown, 1969).

Wu, Lin & Tanaka (1968) have also isolated mutants able to grow on a carbon source not utilized by a wild-type strain. *Klebsiella* (*Aerobacter*) *aerogenes* grows in a minimal medium with the ribitol as carbon source, but is not able to grow on xylitol. Ribitol induces the synthesis of ribitol dehydrogenase and this enzyme has some activity with xylitol as a substrate, but xylitol does not induce its synthesis. Wu *et al.* (1968) obtained a series of mutants of *K. aerogenes* which were able to grow on xylitol and the first mutant X1 was found to be constitutive for ribitol dehydrogenase. From

this mutant they isolated a faster-growing mutant X2 which produced an altered enzyme with a higher affinity for xylitol as a substrate. The apparent K_m for xylitol of the ribitol dehydrogenase from mutant X2 was 120 mM as compared with 290 mM for mutant X1; the corresponding values for ribitol were 25 mM and 31 mM. They obtained a further mutant X3 which was constitutive for a permease system which accepts xylitol but normally transports D-arabitol. Since even the altered ribitol dehydrogenase had a very low affinity for xylitol as a substrate, the active concentration of xylitol by a permease gave this mutant a growth advantage over both X1 and X2. Adaptation to growth on a novel substrate in this case involved structural and regulator genes for ribitol dehydrogenase and an unrelated permease.

Kelly & Clarke (1962) examined the substrate range of the wild-type amidase *Pseudomonas aeruginosa*: it was limited by the size of the amide side chain. The relative rates of hydrolysis of the aliphatic amides were formamide ≪ acetamide < propionamide. The substituted amides glycollamide and acrylamide were hydrolysed at rates intermediate between acetamide and propionamide. We have now shown that butyramide, isobutyramide and lactamide are all hydrolysed at low rates. The *Pseudomonas aeruginosa* B6 mutation results in an enzyme which is able to hydrolyse these latter amides at higher specific rates; this suggests that the substrate binding site of the B enzyme more readily accepts them as substrates. The additional mutations, which allow growth on valeramide, shifted the substrate range even further in favour of amides with larger side chains and in a few cases the specific rate of acetamide hydrolysis had dropped to such an extent that the mutant was unable to grow on acetamide. Extensive physicochemical comparisons have been made between the A and B amidases but no significant differences have yet been detected in overall composition or in the peptide fragments obtained after trypsin hydrolysis or cleavage with cyanogen bromide, in spite of the very clear cut difference in the electrophoretic mobilities of the two enzymes (P. R. Brown, unpublished). The A and B amidases may differ in a single amino acid located at or near the substrate-binding site of the enzyme. However, the alteration might be at another position in the polypeptide chain, producing an alteration in the conformation of the enzyme protein which allowed it to accept butyramide more readily. It would have been interesting to have compared the A and B amidase with the enzymes produced by the v mutants since the latter would be expected to have at least two amino acid alterations. The finding that only partial cross-reaction occurred between extracts of the v mutants and antiserum to the A amidase indicated that there were structural differences between the enzyme proteins. Unfortunately the instability of the V amidases in the cell-free state made it impossible to purify the active enzyme proteins.

Pollock (1965) compared two penicillinases from different strains of *Bacillus licheniformis* and found that there were slight differences in the electrophoretic mobilities, and considerable differences in the enzyme constants. The maximum specific activities of the two enzymes differed by a factor of 6 and the substrate affinities differed also by a factor of 6 but in the opposite direction. This meant that V_{max}/K_m for the two enzymes was almost identical and Pollock (1965) suggested that this represented the physiological efficiency of the enzyme in that at low substrate concentrations which might be encountered in a natural habitat the two enzymes would hydrolyse penicillin at about the same rate. Our A and B amidases have approximately 10-fold differences in respect to both V_{max} and K_m with butyramide in the transferase

reaction. The rate of butyramide hydrolysis by the A enzyme was so low that it was technically difficult to determine these parameters for butyramide in the hydrolase reaction. If ratios of the K_m and V_{max} values for butyramide hydrolase activities of the A and B amidases are comparable with those for the butyramide transferase activities, then the physiological efficiencies of the A and B amidases with respect to growth on butyramide may differ by a factor of about 100.

In our system the mutation-producing strain B6 seems to have resulted in a very marked difference in substrate specificity without significant loss of activity towards its former substrates. We have usually taken acetamide transferase activity as the norm in comparing the activities of mutant enzymes, since this has the highest specific rate of all the reactions known to be catalysed by the wild-type enzyme: amide and ester hydrolysis; acyl transfer, from amides, acids and esters. However, the physiological role of the enzyme during growth on amides depends on the rate of amide hydrolysis and propionamide was found to be hydrolysed more rapidly by suspensions of the B mutants than by the strain producing wild-type enzyme. The K_m for propionamide in the transferase reaction of the B enzyme was less than that of the A enzyme and if we can extrapolate this to the hydrolase reaction, then with propionamide hydrolysis as the norm the B mutant amidase could be said to have a higher physiological efficiency than the wild-type enzyme, together with an alteration in specificity range in favour of higher chain length amides.

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