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Stalk Elongation in Mutants of *Caulobacter crescentus*

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SUMMARY

Morphological mutants of *Caulobacter crescentus*, which undergo extensive stalk elongation when grown on complex medium, were isolated by an indirect selection technique. Wild-type *C. crescentus* is known to produce a similar morphological defect when grown under conditions of severe phosphate limitation. The maximum stalk length of the mutants was 20 μ, as compared with 2 to 3 μ for the parent strains. In these mutant strains, stalk synthesis proceeded in individual bacteria as long as they remained viable. This was in contrast to the wild-type bacteria cultivated on complete medium, where stalk growth reached completion within the first few generations. The mutants had no organic growth factor requirements, but their growth rates were considerably slower than those of the wild-type strains. Two of the mutants showed conditional phenotypic reversion to normal stalk formation when cultivated in the presence of relatively high inorganic phosphate concentrations (10^{-2} M). No ultrastructural defects in the mutants were noted, other than the excessive stalk length. Genotypic reversion rates for back-mutation to short stalk formation were estimated.

INTRODUCTION

The morphogenesis and ultrastructure of vibrioid Caulobacter species have received considerable attention (review by Poindexter, 1964; Poindexter & Cohen-Bazire, 1964; Cohen-Bazire, Kunisawa & Poindexter, 1965; Schmidt & Stanier, 1966). A growing culture of *Caulobacter crescentus* contains motile vibrioid swarmer bacteria possessing a polar flagellum, and non-motile stalked bacteria. The stalked bacterium divides by transverse binary fission, and the swarmer, before division, develops its stalk at a location corresponding to the site of attachment of the flagellum (Stove & Stanier, 1962; Poindexter, 1964). Ultrastructural investigations demonstrated that the stalk is enclosed by a multi-layered wall which is continuous with the bacterial cell wall, and that the cytoplasmic membrane continues into the stalk to enclose its core (Poindexter, 1964; Poindexter & Cohen-Bazire, 1964). This core is composed of a membranous material which extends from a differentiated membranous region at the pole of the bacterial cell. The polar membranous region associated with the stalk is structurally distinct from the mesosomes which originate from the cytoplasmic membrane and which also may occur in this region of the bacterium (Cohen-Bazire *et al.* 1965). Stalk growth occurs at the juncture of the stalk with the bacterial cell, and the stalk wall is a relatively inert, non-growing structure (Schmidt & Stainer, 1966).

These studies have raised interest in the biochemical activities which accompany and control stalk development. In one approach to this problem, mutant strains with

aberrations in their stalk development have been sought for biochemical characterization. This paper reports the isolation of mutants of *Caulobacter crescentus* which undergo extensive stalk elongation when cultivated on standard complex growth medium. These mutants will be referred to by the phenotypic abbreviation, Skl, which stands for stalk length. The morphology, ultrastructure and growth characteristics of several Skl mutant isolates are described here.

METHODS

Bacteria and cultural conditions. Caulobacter crescentus strains CB15 (ATCC 19089) and CB1 were obtained from Dr Jeanne Poindexter. The standard complex growth medium (PYE) and the glucose + imidazole defined medium (G + I) were as previously described (Poindexter, 1964; Schmidt & Stanier, 1966). Cultures were incubated at 30°; liquid cultures were grown in flasks in a rotary shaker water bath (New Brunswick Scientific, Model G 76) at 130 rev./min. to provide adequate aeration.

Isolation of mutants. Mutagen treatment of *Caulobacter crescentus* wild-type cultures with ultraviolet radiation or ethylmethane-sulphonate (EMS) was used. For u.v.-radiation, an exponential culture in PYE broth was diluted 1/10 in G + I medium to give about 5×10^7 bacteria/ml. The bacteria were irradiated 3 to 4 min. at a distance of 14 cm. with a Mineralite USV-11 lamp. This treatment gave 1 to 0·1 % survival. The irradiated cultures were diluted in PYE broth and PYE spread plates prepared.

A method adapted from Strauss (1962) and Paleroni & Stanier (1964) was used in EMS treatments. Two ml. of bacterial culture in the late exponential stage of growth were harvested and resuspended in 1 ml. of 0·03 M-phosphate buffer (pH 7). The buffered suspension was added to 1 ml. of 2 % (v/v) EMS (Eastman Organic Chemicals) in 0·1 M-phosphate buffer (pH 7). The mixture was incubated at 30° for 70 to 90 min. to obtain 1 to 0·1 % survival. The EMS-treated bacteria were diluted into 5 % (w/v) $Na_2S_2O_3$ to destroy residual mutagen. Subsequent dilutions were made in PYE broth, and 0·1 ml. samples were plated on PYE agar. After growth appeared, the colonies were used in preparing PYE agar patch plates to obtain sufficient growth, originating from individual colonies, for further observation. After growth of patch areas had occurred, bacteria from the patches were examined with a phase-contrast microscope at a magnification × 1250. When morphological mutants were detected, bacteria from corresponding patch areas were streaked on PYE agar.

Growth rate, viability, and biochemical determinations. Growth rates were estimated turbidimetrically with a Klett–Summerson photoelectric colorimeter with a no. 66 filter. Five ml. of inocula from PYE or G + I liquid cultures in the exponential phase of growth were added to respective flasks containing 30 ml. of PYE or G + I broth; growth experiments continued for 12 to 15 hr.

To determine % viable forms in late exponential phase PYE cultures, viable counts (colony forming units/ml.) were obtained from PYE spread plates inoculated with appropriate dilutions of the culture; direct counts of bacteria were done by using a Petroff-Hausser counting chamber. Rosettes were counted as one colony-forming unit.

The poly-β-hydroxybutyrate content was estimated gravimetrically (Williamson & Wilkinson, 1958) on Caulobacter cultures grown in G + I medium containing 10^{-3} M-inorganic phosphate.

Preparations for electron microscopy. The method of Brenner & Horne (1959) as

modified by Bradley (1962) was used for negative stains. A suspension of bacteria (10^8/ml.) was prepared in distilled water. A drop of the suspension was placed on a clean glass slide and mixed with a drop of 2% (w/v) sodium phosphotungstate containing 0·4% sucrose. A formvar-coated carbon-stabilized grid was dipped in the suspension of stained bacteria and allowed to dry before electron microscopic observation.

For preparation of thin sections, a modification of the method of Ryter & Kellenberger (1958) was used. The veronal+acetate buffer used in fixation was at pH 6·8. Bacteria were fixed in 1% osmium tetroxide for 2·5 hr. The embedding medium was Vestopal (Polysciences, Inc.), polymerized at 60° for 48 hr. Sections were obtained with a MT-1 ultramicrotome (Sorvall), post-stained with lead citrate (Reynolds, 1963), and observed with a Philips 100 B electron microscope operated at 60 kV.

Genotypic reversion rate estimation. The Poisson distribution method (Luria & Delbrück, 1943; Witkin, 1950) was used for approximation of the genetic reversion rate of several *Caulobacter crescentus* Skl mutants to revertant forms with stalks of normal length. The revertants were detected since they grew faster and formed larger colonies than the Skl strains; their presence was verified by microscopic examination. The fraction of apparent non-revertant colonies among total colonies (P_0) on six PYE spread plates was determined for each of five Skl strains. Each colony was considered to represent an independent culture. The average number of bacteria per colony (N) was determined by making direct microscopic counts of bacteria from Skl colonies suspended in 0·1 ml. PYE broth. This adaptation of the Poisson mutation rate estimation makes the assumptions that no revertants were present among the Skl bacteria originally plated, and that revertants which occurred during the growth of the colonies would give rise to larger and distinctive revertant colony forms.

Extracts of Caulobacter crescentus. Late exponential phase cultures of wild-type *C. crescentus* CB1 and CB15 were treated ultrasonically for 1 min. with a cooled probe (Model S 75, Branson Instruments, Inc.). The suspensions were then centrifuged at 3000 *g* for 10 min. The supernatant fluids were passed through a Millipore type HA filter and the filtrates used in phenotypic reversion experiments.

Phenotypic reversion determinations. G+I or PYE agar media containing added inorganic salts (Na_2HPO_4 and KH_2PO_4, 2×10^{-4} to 2×10^{-2} M; KCl, NaCl, $CaCl_2$, $MgCl_2$ or $MgSO_4 . 7H_2O$, all at 2×10^{-2} M), filtrates of wild-type bacterial extracts (10%, v/v), pantoyl lactone (Nutritional Biochem. Corp., $10^{-4} \times 3$ to 10^{-1} M), and calcium D-pantothenate (Calbiochem, 0·1 to 10 μg./ml.) were prepared. Skl strains were streaked on the phenotypic reversion (PR) media, and after growth, the bacteria examined microscopically. When a majority of the bacteria possessed stalks much shorter than the usual Skl morphology, bacteria from the PR plate were re-streaked on PYE agar and after growth had occurred, their morphology examined. A return to Skl morphology on the PYE plate indicated that conditional reversion, rather than back mutation, had occurred on the PR medium. A second method of testing filtrates of bacterial extracts was used: 0·1 ml. of extract was added to 'penicylinders' (Fisher Scientific Co.) which were placed over the regions of Skl inocula on PYE agar plates.

RESULTS

Isolation of Skl mutants of Caulobacter crescentus

Several Skl mutants of *Caulobacter crescentus* which undergo extensive stalk elongation on PYE medium have been isolated by an indirect selection technique. The morphological aberration in these mutant strains is readily detectable by using high-resolution phase-contrast microscopy, and is comparable to the morphological effect of inorganic phosphate starvation on wild-type *C. crescentus* strains (Schmidt & Stanier, 1966). The Skl mutant strains, grown on PYE medium without deliberate phosphate starvation, developed stalks with maximum lengths 12 to 20 μ. Not all of the stalks were of maximum length in Skl cultures that had not yet reached the stationary phase of growth, since swarmer bacteria continually appeared with each new generation and produced stalks that were shorter than those of bacteria which had originated earlier in the culture. This variability in stalk lengths was also observed in growing cultures of phosphate-limited wild-type *C. crescentus* (Schmidt & Stanier, 1966).

Table 1. *Source of Skl mutants of Caulobacter crescentus*

Parent strain	Mutagen	Mutant strain designation
CB15	EMS	Skl 418
CB15	EMS	Skl 1413
CB15	U.v.	Skl UV23
CB1	EMS	Skl 219
CB1	EMS	Skl 220
CB1	EMS	Skl 221

Six Skl isolates were chosen for further study (Table 1). In the absence of a direct selection technique for Skl mutants, most of the mutagen experiments gave few, if any, Skl isolates. The detection of Skl strains was aided somewhat by the slower growth of the mutants, giving rise to Skl colonies that were smaller than those of wild-type bacteria. All unusually small or minute colonies were given particular attention on the primary isolation plates. However, this was not a specific differential character since many mutations besides those which affected stalk length also affected colony size. The colour of Skl colonies differed from that of wild-type strains. Skl colonies are chalk white; wild-type colonies are cream-coloured. However, in examining the very small colonies on the primary isolation plates, this was not a particularly obvious differentiation aid.

Morphology

Negatively stained preparations of wild-type *Caulobacter crescentus* strains CB1 and CB15 are shown in Pl. 1, fig. 1, 2. Similar preparations of Skl mutants 418 and UV23 (derived from CB15) and Skl mutant 220 (from CB1) are shown in Pl. 1, fig. 3, Pl. 2, fig. 4, 5. The excessively long stalks of the Skl strains contained relatively few crossbands. Three or four crossbands appeared in Skl bacterial stalks; wild-type bacteria from the stationary phase of growth often possessed as many. The Skl mutants frequently had a somewhat aberrant cellular morphology. The bacteria appeared slightly elongated and serpentine, and occasionally seemed to encounter difficulty in completing the binary fission process. However, filamentous bacteria were seldom encountered in Skl cultures, and daughter swarmer bacteria were notably present in

growing Skl cultures. Poly-β-hydroxybutyric acid deposits, which appeared as electron-light intracellular granules, were frequently seen in Skl bacteria, but occurred less extensively than in the phosphate-limited wild-type *C. crescentus* (Pl. 3, fig. 6).

Poly-β-hydroxybutyric acid accumulation

Extensive poly-β-hydroxybutyric acid deposition is characteristic of unbalanced growth in Caulobacter, particularly under conditions of nitrogen starvation (Poindexter, 1964) or phosphate limitation (Schmidt & Stanier, 1966). Some of the Skl strains appeared to accumulate substantial quantities of poly-β-hydroxybutyric acid when observed in light and electron microscopic preparations. Gravimetric determinations for this reserve compound were made on the Skl and wild-type strains. The only Skl isolate with a markedly increased poly-β-hydroxybutyric acid content was UV23 (150 μg./mg. dry wt, compared to 100 μg./mg. for the parent strain CB15). The other Skl strains had amounts which compared closely to that of their parent strain.

Ultrastructure

Electron micrographs of thin-sectioned Skl mutants were compared with those of similarly prepared wild-type organisms. No particularly unusual ultrastructural features of the Skl strains were noted (Pl. 3, fig 7; Pl. 4, fig. 8). Thin sections of Skl bacteria along the entire long axis, including the excessively long stalks, were seldom obtained. Thin sections of very long stalks were occasionally observed, and except for their excessive length, ultrastructural organization was similar to that of the wild-type Caulobacter. The polar differentiated membranous region, from which stalk growth is thought to begin, was comparable in Skl and wild-type strains.

Growth and viability characteristics

The growth rates of the Skl mutants in PYE medium were considerably slower than those of the parental strains and the % viable bacteria in late exponential phase PYE cultures were lower for Skl bacteria than for wild-type cultures (Table 2). The loss of viability in older Skl cultures compared with that in phosphate-limited wild-type cultures.

Table 2. *Growth characteristics of Caulobacter crescentus strains*

Strain	Growth medium	Doubling time (hr)	Viability (%)
CB15 wild-type	Peptone + yeast-extract (PYE)	1·8	Not done, due to rosette formation
CB15 wild-type	Glucose + imidazole + 10^{-3} M-phosphate	3	100
CB15 wild-type	Glucose + imidazole no added phosphate	5	46
CB15 Skl 418	PYE	10	70
CB15 Skl 1413	PYE	5	49
CB15 Skl UV23	PYE	5	80
CB1 wild-type	PYE	2	Not done, due to rosette formation
CB1 Skl 219	PYE	8	48
CB1 Skl 220	PYE	8	49
CB1 Skl 221	PYE	6	41

Genotypic reversion

Skl strains frequently underwent back mutations to morphological forms having stalks of normal length and growing faster than the Skl mutants. The revertants often were not identical phenotypically with the original wild type, and frequently showed aberrations in cell division. Bacterial filament formation was commonly observed among the revertants. The occurrence of back mutation necessitated occasional re-isolation of Skl strains maintained in vegetative culture. Twenty % or less of the isolated colonies of Skl strains gave detectable revertants, as determined from larger colony size and subsequent microscopic examination. Results of reversion rate estimations for five Skl strains are given in Table 3. The reversion rate of strain UV23 was not determined because its revertant colonies did not differ significantly in size from Skl colonies. The other five strains gave revertant colonies that were easily detectable (Pl. 4, fig. 9). In the method applied here, mutations resulting in reversion that occurred late in the growth of Skl colonies might not result in detectable colony differences, and the reversion rate obtained probably appears to be lower than might actually be the case. Because of the lack of a direct selective technique, the mutation rate of normal wild type *Caulobacter crescentus* to Skl mutants was not determined.

Table 3. *Genotypic reversion rates and frequencies of Skl mutants of Caulobacter crescentus*

Strain	Estimated reversion rate* (no. mutations/ cell/ generation)	Frequency of occurrence of revertant colonies
CB15 Skl 418	$1{\cdot}1 \times 10^{-8}$	0·20
CB15 Skl 1413	$1{\cdot}5 \times 10^{-8}$	0·23
CB1 Skl 219	$6{\cdot}9 \times 10^{-9}$	0·15
CB1 Skl 220	$7{\cdot}5 \times 10^{-9}$	0·12
CB1 Skl 221	$6{\cdot}3 \times 10^{-9}$	0·12

* $a = -(\ln 2)(P_0)/N$, where a = the mutation rate, P_0 = the proportion of non-revertant colonies and N = the average number of bacteria/Skl colony.

Conditional phenotypic reversion

Of the several inorganic and organic compounds tested, only inorganic phosphate at concentrations greater than 10^{-2} M gave reproducible phenotypic reversion with any of the Skl mutants. Skl strains UV23 and 1413 responded conditionally to high phosphate concentrations, producing stalks that were only 5 to 10 μ in length, then returned to typical Skl morphology when subcultured on PYE medium. The Skl mutants derived from strain CB1 also showed a slight degree of conditional reversion on media with 10^{-2} M-phosphate. However, stalk lengths of the Skl mutants of CB1 in high phosphate medium were usually greater than 10 μ so the repression of stalk elongation by phosphate was not as effective in these CB1 mutants. Skl strain 418, derived from CB15, did not respond to phosphate or to any of the other compounds tested. Filtrates of wild-type bacterial extracts allowed no phenotypic reversion of the Skl strains.

DISCUSSION

The possibility that some of the Skl mutants obtained may possess defects in phosphate uptake or metabolism is suggested by the conditional phenotypic reversion of these mutants to forms with almost normal stalk length when grown in the presence of high phosphate concentrations. However, some of the Skl isolates responded only slightly or not at all to the phosphate concentrations tested, implying that control of stalk formation may be affected by factors other than the availability of inorganic phosphate.

The mutations resulting in Skl morphology are somewhat detrimental to the growth and survival of the bacteria. Despite their decreased viability and slower growth rates, the Skl mutants of *Caulobacter crescentus* appear to offer an opportunity for further study of the control of bacterial stalk formation.

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EXPLANATION OF PLATES

Abbreviations used in figures: S, stalk; *c*, core of stalk; *cb*, cross-band in wall of stalk; *f*, flagellum; *p*, poly-β-hydroxy-butyric acid granule; R, revertant colony.

PLATE 1

Caulobacter crescentus strains, cultivated in PYE medium, and negatively stained with phosphotungstate.

Fig. 1. CB15 wild-type. Magnification × 10,500.

Fig. 2. CB1 wild-type. Magnification × 10,500.

Fig. 3. CB15 Skl mutant 418. The core of the stalk can be discerned. Magnification × 17,500.

PLATE 2

Skl mutants of *C. crescentus*, cultivated in PYE medium, and negative-stained with phosphotungstate.

Fig. 4. CB15 Skl mutant UV 23. Magnification, × 9000.

Fig. 5. CB1 Skl mutant 220. Magnification, × 14,500.

PLATE 3

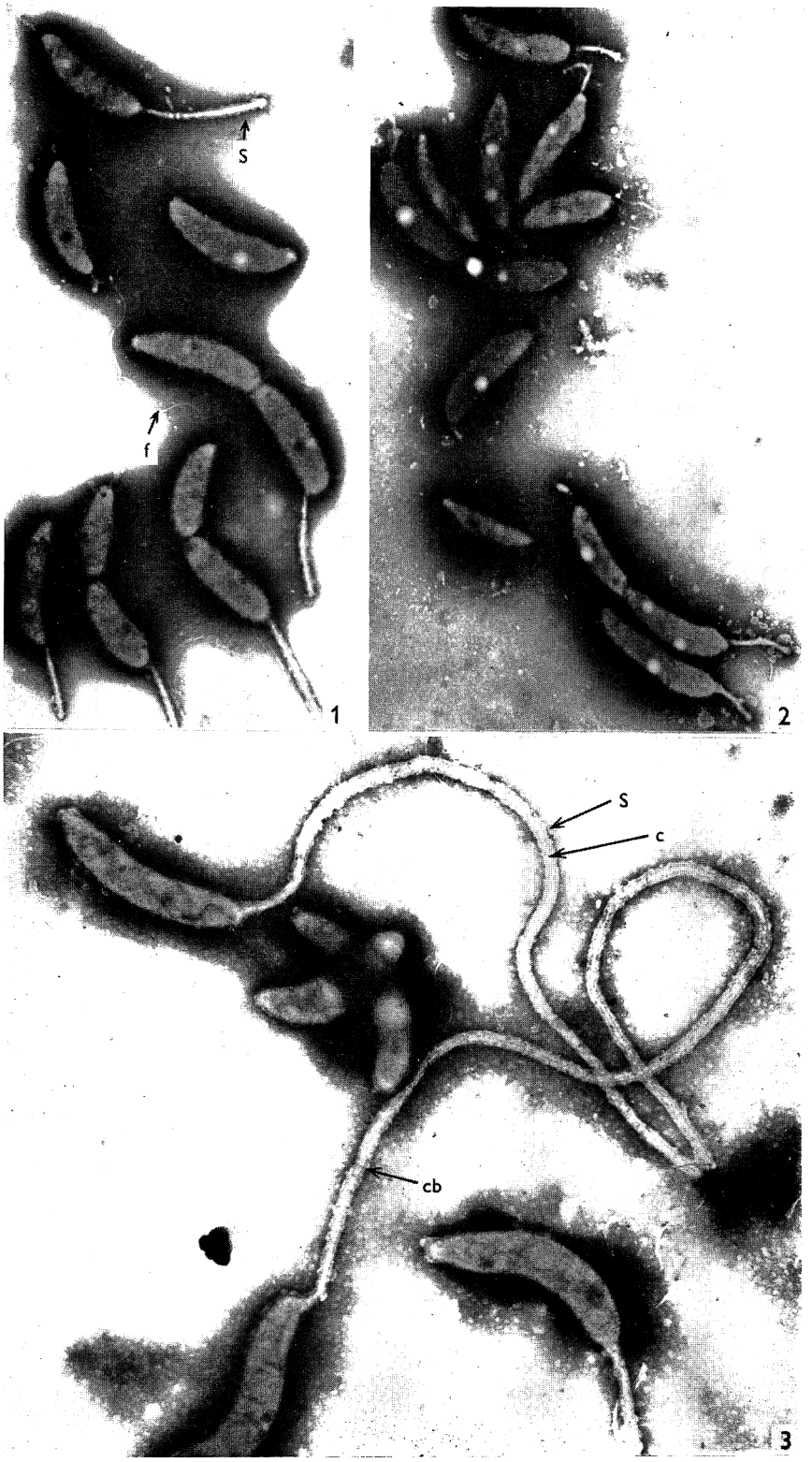
Fig. 6. *C. crescentus* CB15 wild-type, grown under conditions of phosphate starvation in G-I medium, with 10^{-5} M-phosphate. The bacteria contain massive accumulations of poly-β-hydroxybutyric acid. Magnification × 10,000.

Fig. 7. Thin section of mutant Skl 418, fixed with osmic acid, embedded in Vestopal, and post-stained with lead citrate. Magnification, × 46,200.

PLATE 4

Fig. 8. Thin section of mutant Skl UV 23. Magnification × 42,000.

Fig. 9. A PYE agar spread culture of Skl mutant 418 after incubation for 72 hr. Examination of the bacterial morphology of the colonies with a phase-contrast microscope revealed that the seven large prominent colonies contained predominantly revertant bacteria with stalks of normal length. The small colonies consisted of caulobacters with Skl-mutant morphology.



J. M. SCHMIDT

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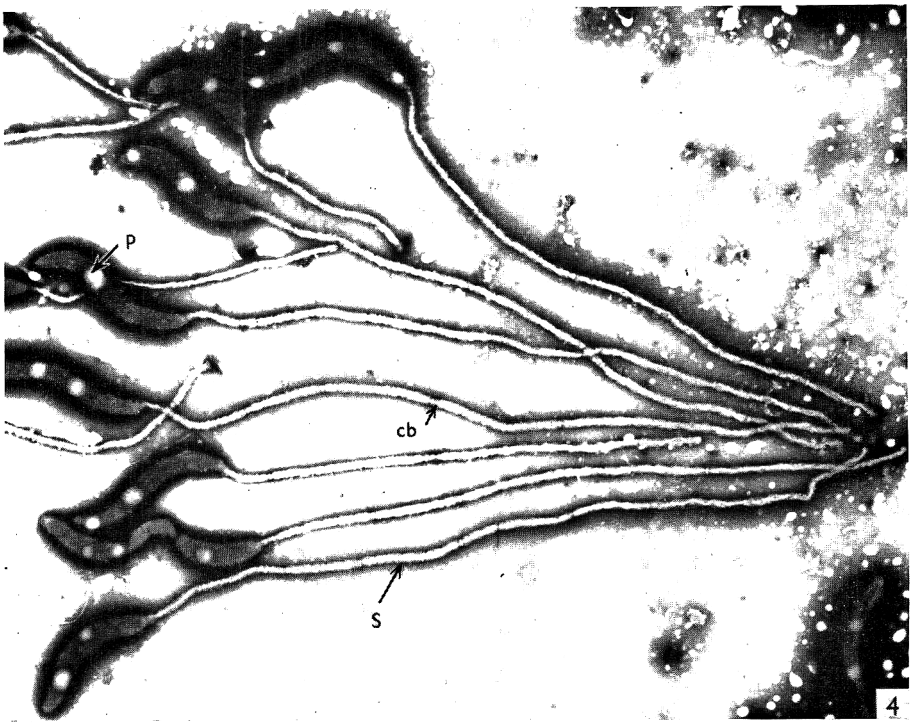
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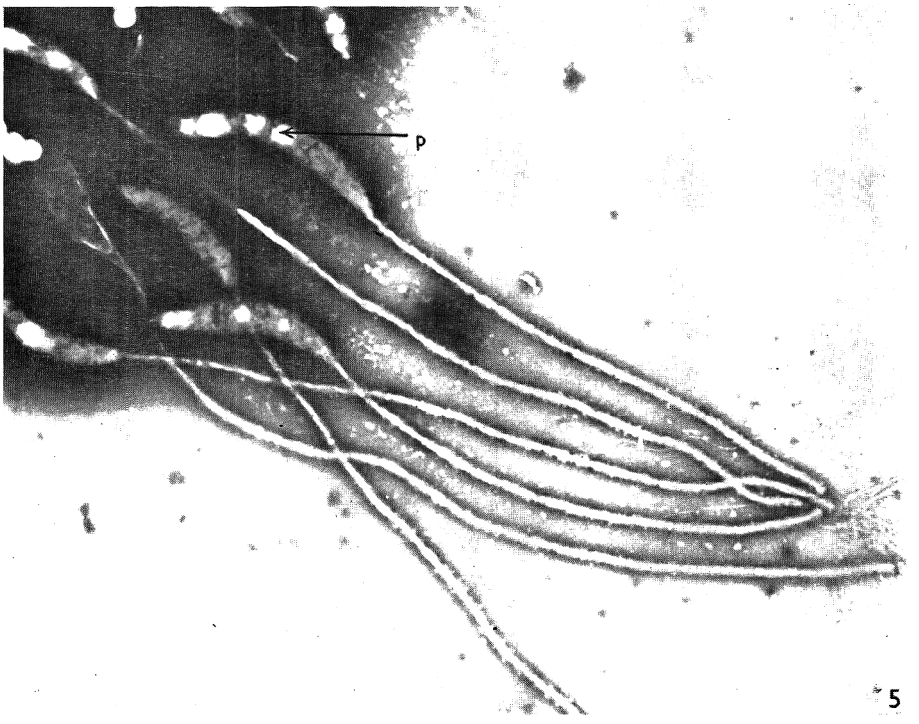
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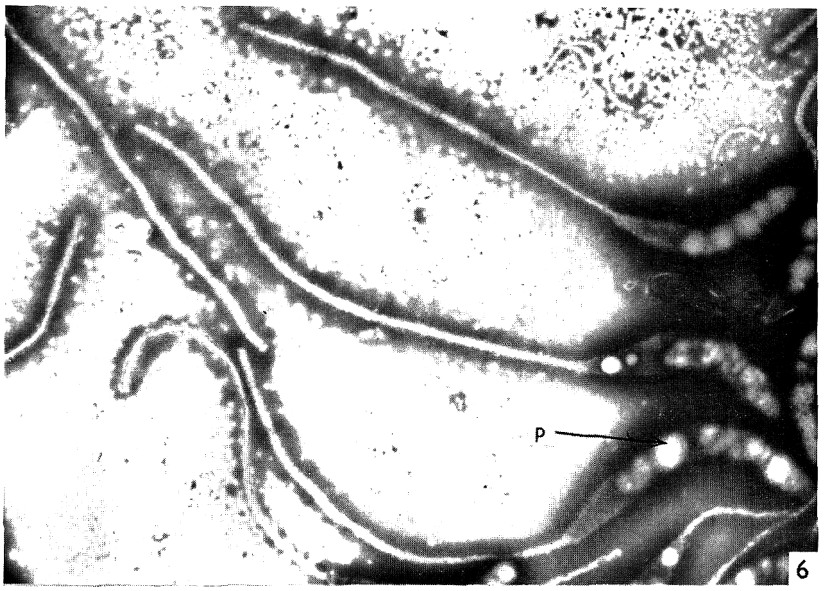
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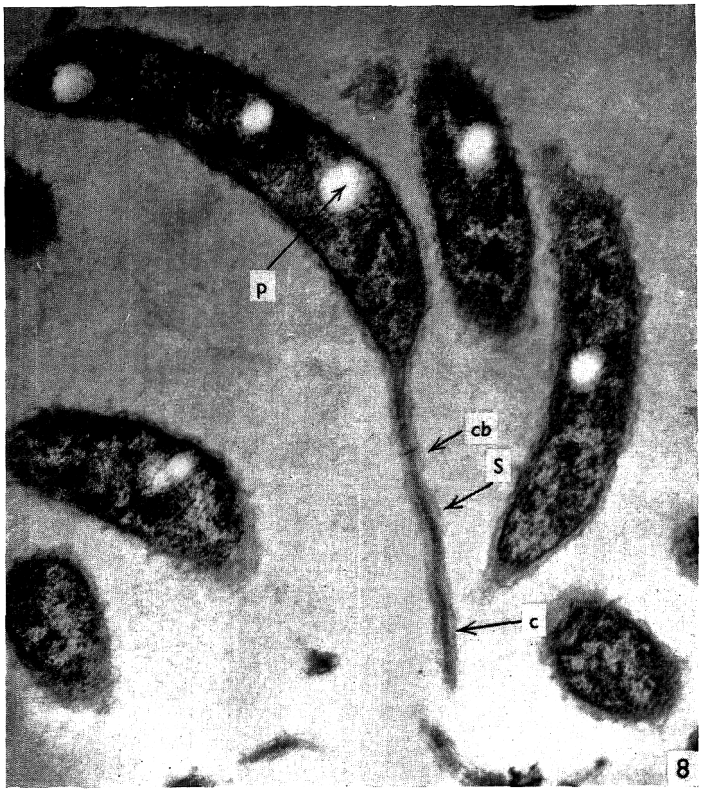
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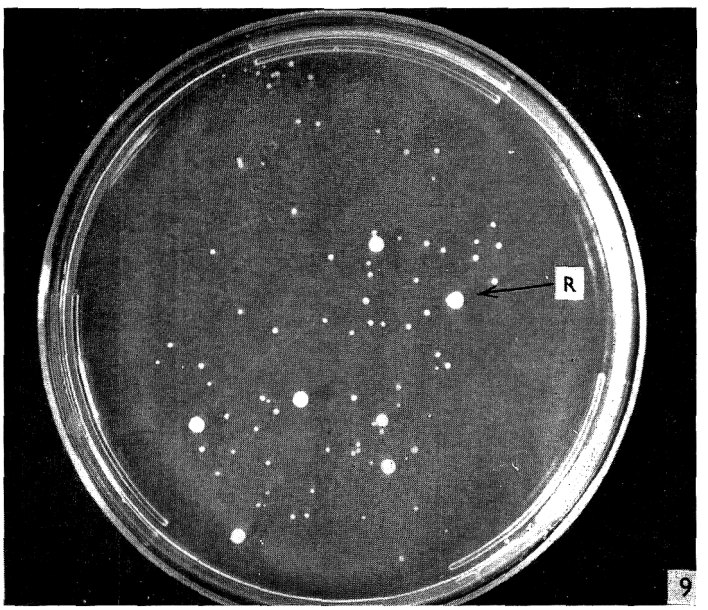
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A Numerical Taxonomic Study of 100 Isolates of *Corynebacterium pyogenes*

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SUMMARY

An Adansonian analysis was made on 100 isolates of *Corynebacterium pyogenes* with the aid of a digital computer. A wide variety of tests was used to define the isolates. The resulting dendrogram showed that *C. pyogenes* was a 'good species', with the 100 isolates related at high similarity values. No close affinity between biotype and host-species or between biotype and lesion was found, apart from the disposition within two adjacent groups of all of the isolates which originated from Swedish bulls.

INTRODUCTION

Corynebacterium pyogenes is a common pathogenic bacterium for all the larger domesticated animals. It is responsible for much economic loss and is present, as a primary or secondary invader, in almost all purulent conditions of cattle, sheep and pigs. The purpose of the present work was to study in detail a group of isolates of *C. pyogenes* isolated from a variety of animal hosts and morbid processes, and to utilize the information so obtained to estimate numerically with the aid of an electronic digital computer, the over-all similarity of the isolates. They were then to be disposed in a numerical classification, to ascertain whether any correlation obtained between such a classification and the species of host or the lesion of origin.

METHODS

A collection of 100 isolates of *Corynebacterium pyogenes* (53 from disease processes in cattle, including 19 strains from Sweden; 22 from pigs; 21 from sheep; three from wild rabbits, and one from a horse) was used. The isolates were maintained on 5% (v/v) blood agar, subcultured at weekly intervals and a new series of cultures was prepared monthly from a stock of freeze-dried cultures. Each isolate was subjected to the following tests:

A. *Morphology*. (1) Mean dimensions of bacteria grown under standard conditions. (2) Presence of basophilic cytoplasmic inclusions. (3) Evidence of extreme pleomorphism. (4) Presence of metachromatic granules.

B. *Biochemistry*. (1) Fermentation tests on arabinose, rhamnose, xylose, fructose, galactose, glucose, mannose, cellobiose, lactose, maltose, sucrose, trehalose, raffinose, dextrin, glycogen, inulin, starch, adonitol, dulcitol, erythritol, glycerol, mannitol, sorbitol, amygdalin, arbutin, coniferin, salicin. (2) Production of acetylmethylcarbinol, catalase, cytochrome (Deibel & Evans, 1960), coagulase, lysine and arginine decarboxylase, dihydroxyacetone, β-galactosidase, hydrogen peroxide, indole, lecithi-

nase, lipase, oxidase, phenylalanine deaminase, phosphatase, Tween hydrolase, reduction of methylene blue, methyl-red test (Barritt modification) and citrate utilization test (Koser, 1923).

C. *Growth tests.* (1) Type of deposit in fluid media; turbidity of standard suspensions after incubation for 18 hr. (2) Production of soluble haemolysin.

D. *Physical tests.* (1) Slide auto-agglutination, in saline (pH 6·8); tube agglutination at various pH values; resistance to high salt concentration (6%); growth at 46°; growth in lauryl sulphate broth (0·01%); survival of culture at 4°; growth in potassium cyanide medium (0·007%); growth in serum agar plates containing pyronin (0·001%), methyl-violet (0·002%) basic fuchsin (0·004%) or thionine (0·0033%); growth on MacConkey medium (with and without enrichment with 3% serum); growth on bismuth sulphite agar. (2) Sensitivity to bacitracin (15 units), chloramphenicol (10 μg.), sulphafurazole (100 μg.), neomycin (10 μg.), nitrofurantoin (200 μg.), penicillin G (1·5 units), polymyxin B (100 units), streptomycin (10 μg.), methicillin (10 μg.), colistin (50 μg.), fucidic acid (10 μg.). kanamycin (5 μg.), novobiocin (5 μg.), cloxacillin (5 μg.), ampicillin (2 μg.).

Analysis of the results obtained for the hundred strains was done according to Adansonian principles of bacterial taxonomy modified according to Sneath (1962) for the purpose of computer analysis. The main endeavour was to distribute strains into groups or clusters, whereby each was composed of mutually similar strains. Tests where every strain gave a positive, or a negative result, were not incorporated in the data table. The results of the remaining tests (62), which gave variable results, were coded according to the requirements of Gower's CLASP programme, and estimation of coefficients of mutual similarity of the strains was made by J. C. Gower of Rothamsted Experimental Station. The programme used is capable of accepting information of three kinds—dichotomies (+ or −), quantitative (e.g. morphology measurements) and qualitative (e.g. litmus milk test, where mutually exclusive reactions, unrelated by rank, occur). Calculation of coefficients of similarity (S) were calculated and expressed in the form of a similarity matrix indicating the S values for each strain relative to every other. The method of calculation of S values in this programme does not take into account negative matches, for the reasons advanced by Sneath (1962). Cluster grouping was done by the technique of 'single link listing', a method devised by Dr J. E. Smith (personal communication, 1965) and described and successfully used by Hussaini (1965).

Diagrammatic representation of the groupings was by means of a dendrogram (Fig. 1) which rendered affinities of strains and clusters of strains apparent.

RESULTS

The computer calculated values of S ranged from 55% to 85%. Those values are indicated in the dendrogram (Fig. 1) as horizontal lines at the appropriate value, linking the isolate, or group stems. Isolates which were joined in the dendrogram at values of 85% S, e.g. no. 87:85 were obviously closely related, whereas isolates linked at 55% were only distantly related. The percentage similarity of 68% was selected as a suitable value for definition of major groups, since at that value 93 of the 100 isolates were encompassed within five major groups.

Table 1 indicates the distribution of isolates from different host species among the

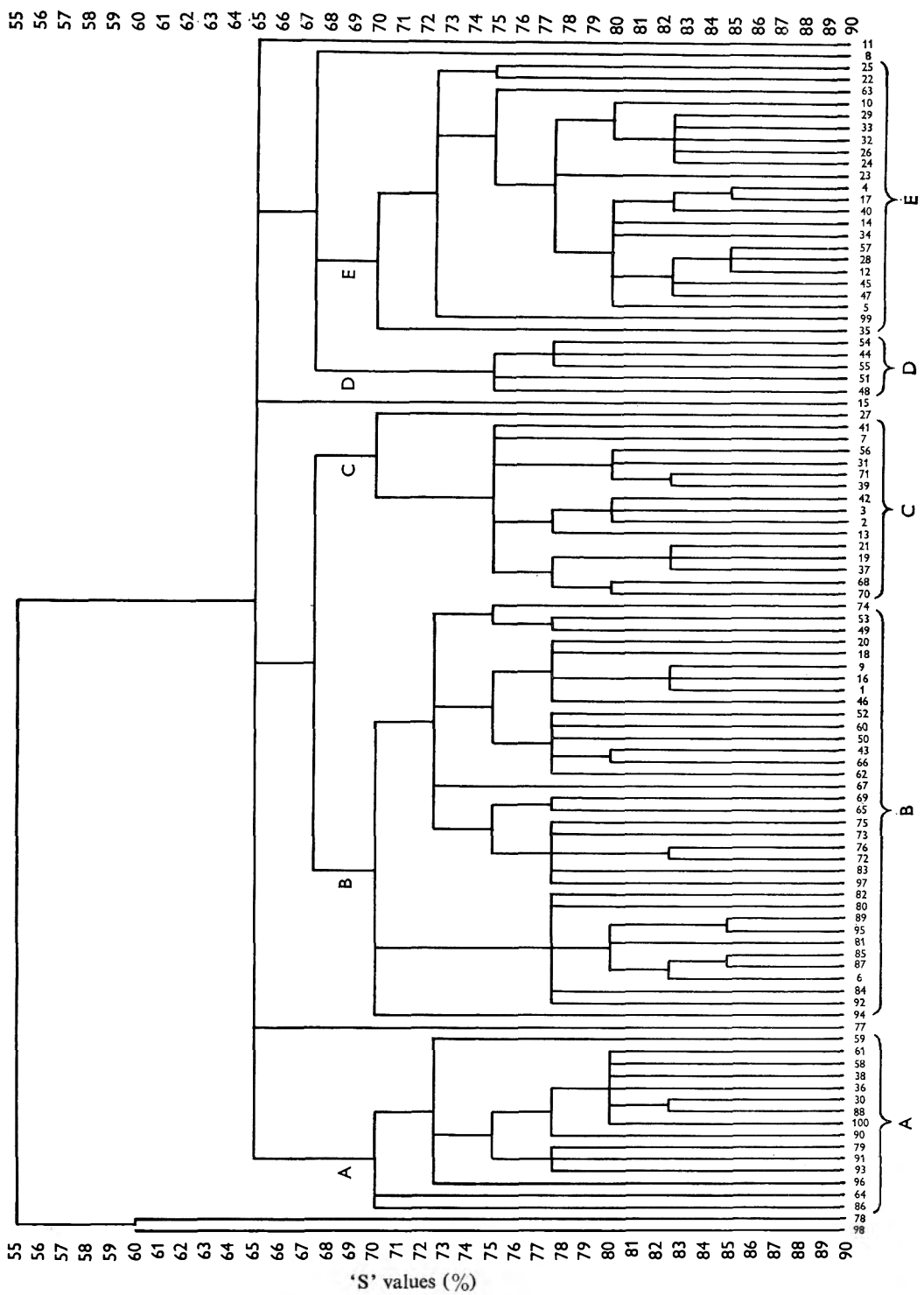

Fig. 1. Dendrogram derived from computed S values for 100 isolates of *Corynebacterium pyogenes*. The five main groups at S value 68 % are indicated beneath the isolate numbers on the base-line.

dendrogram defined groups. The most striking feature of Table 1 is that of the 15 isolates in group A only 1 was not of bovine origin. The distribution of isolates in groups B and E was not considered significant, but in groups C and D the percentage of porcine isolates was much higher (47 and 60%) than would accord with random distribution. Isolates from sheep were distributed evenly throughout the groups, except that there were no ovine isolates in group A.

Table 2 shows that while isolates from most parts of the body occurred uniformly throughout the groups, those recovered from the male genital tract were entirely confined to groups A and B. The significance of that finding is difficult to assess because those isolates comprised 17 of the 18 isolates from Sweden, and the other isolate from that country, an ovine one (77) appeared in the dendrogram as an intermediate between groups A and B.

Table 1. *Distribution of 100 Corynebacterium pyogenes isolates amongst the computer-defined groups*

Host species	No. of isolates	Computer-defined groups A	B	C	D	E	Miscellaneous
Ox	53	14 (94)	20 (57)	3 (20)	0 (—)	12 (52)	4 (57)
Sheep	21	0 (—)	8 (23)	4 (27)	1 (20)	7 (30)	1 (14)
Pig	22	1 (6)	5 (14)	7 (47)	3 (60)	4 (17)	2 (29)
Others	4	0 (—)	2 (6)	1 (7)	1 (20)	0 (—)	0 (—)
All species	100	15	35	15	5	23	7

The number of isolates is given (followed in parentheses by the percentage) in relation to the number of isolates in each computer-defined group.

Table 2. *Distribution of 100 Corynebacterium pyogenes isolates from different organs or pathological conditions amongst the computer-defined groups*

Organ or pathological condition	No. of isolates	Computer-defined groups A	B	C	D	E	Miscellaneous
Male genital tract	17	6 (40)	11 (31)	0 (—)	0 (—)	0 (—)	0 (—)
Female genital tract	5	1 (7)	1 (3)	2 (13)	0 (—)	1 (4)	0 (—)
Mastitis	21	5 (33)	6 (17)	0 (—)	0 (—)	7 (30)	3 (44)
Nephritis	3	1 (7)	1 (3)	1 (7)	0 (—)	0 (—)	0 (—)
Arthritis	4	0 (—)	1 (3)	2 (13)	0 (—),	1 (4)	0 (—)
Respiratory tract including pneumonia	16	0 (—)	6 (17)	4 (27)	2 (40)	4 (17)	0 (—)
Abscesses	15	2 (13)	5 (14)	1 (7)	2 (40)	3 (13)	2 (28)
Lymph-node lesions	11	0 (—)	1 (3)	2 (13)	1 (20)	5 (22)	2 (28)
Unknown	8	0 (—)	3 (9)	3 (20)	0 (—)	2 (9)	0 (—)
All sources	100	15	35	15	5	23	7

The number of isolates is given (followed in parentheses by the percentage) in relation to the number of isolates in each computer-defined group.

DISCUSSION

The results of this work indicate that the group of 100 isolates of *Corynebacterium pyogenes* studied was a closely knit group possessed of many features common to all isolates. The results of the computer analysis show that *C. pyogenes* merits the description 'good species' since all the isolates used were linked at high similarity values similar to those obtained, for example, by Smith & Thal (1965) with the genus *Pasteurella.*

Cluster analysis of the computed 'S' values indicated close affinity between the 100 *Corynebacterium pyogenes* isolates used here, but did not define any marked relationship between biotype, and lesion of origin (with the exception of the confinement of isolates from Swedish bulls to groups A and B). This suggests that, in Britain at least, biotypes are not limited to a single host or lesion, and that cross-infection among sheep cattle and pigs, as well as infection from feral hosts such as wild rabbits, is perfectly feasible.

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The Metabolic Characterization of the Ciliate Protozoon *Eudiplodinium medium* from the Rumen of Buffalo

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SUMMARY

An oligotrich protozoon identified as *Eudiplodinium medium* was separated in pure culture and inoculated into the rumen of an isolated ciliate-free buffalo (*Bos bubalus* L.) calf to act as a continuous source of the organism. Analysis of the protozoa showed that a single organism contained on average $3{\cdot}35 \pm 0{\cdot}003$ μg. protein; the carbohydrate fraction (49·1 % of dry matter) gave on hydrolysis 60·4 % glucose. Growth rate of the protozoa *in vitro* was dependent on food concentration. The number of protozoa was doubled in 2 hr at a substrate concentration of 0·23 mg./protozoon; at lower concentrations the doubling time was greater; at higher concentrations (0·32 mg./protozoon) the organisms burst.

Eudiplodinium medium did not metabolize simple sugars; it utilized starch, amylopectin, amylose and showed highest activity with hemicelluloses. Xylans were degraded faster than arabans; xylans gave relatively lower amounts of volatile acids than did arabans; acetic and butyric acids predominated, formic acid and traces of propionic and lactic acids were found but no succinic acid. No cellulose digestion took place during incubation for 12 hr; with longer periods of incubation with cellulose the organisms died. Ammonium salts, urea and amino acids were not used by the protozoa; proteolytic activity was observed towards gelatin, casein and gluten in descending order of activity. Cell-free extracts of *E. medium* showed activities towards carbohydrates as did whole organisms; xylanase activity was greatest. This enzyme was purified 28-fold; it had an optimum pH of 7·5 and an optimum temperature of 43°.

INTRODUCTION

The present work is concerned with the isolation of a ciliate oligotrich protozoon, *Eudiplodinium medium*, from the rumen of buffalo (*Bos bubalus* L.). Under similar conditions of feeding, the numbers of this ciliate were greater in buffalo than in cow. The metabolism of this organism was then studied.

METHODS

Isolation of Eudiplodinium medium. Unsuccessful attempts were made to isolate this oligotrich by fractional sedimentation from long columns filled with buffer (Oxford, 1951); the sedimented organisms were contaminated with Entodinium. Isotricha species were destroyed by adding mannose (Abou Akkada & Howard, 1960) to the medium. It was soon realized that Hungate medium (1942) was selective for Eudi-

plodinium species. A strained rumen sample containing a complete protozoan population, obtained from a 4-month-old calf, was diluted 1/10 by Hungate medium. Rye grass in Hungate medium was replaced by barley fodder (harvested at 30 days), dried and added to a final concentration of 0·08 % (w/v). The medium was renewed twice daily by sucking off the upper half of the liquor. Within 7 days, types of protozoa other than *E. medium* and *E. neglectum* had disappeared. *E. medium* was predominant in most inocula; *E. neglectum* did not survive the competition and disappeared. The pure culture of *E. medium* was used for inoculating an isolated ciliate-free buffalo calf so that it only harboured this oligotrich, which reached a reasonable count within 10 days from inoculation. The calf was fed an early weaning concentrate mixture (Khoury, Ahmed & El-Shazly, 1967) and a green fodder *Lolium pereniale*.

Preparation of suspensions of washed protozoa. Rumen samples were withdrawn from the experimental calf before feeding and the protozoa separated and washed as recommended by Oxford (1951). Chloramphenicol (50 μg./ml.) was added to the last washings to decrease the number of bacteria; complete elimination of bacteria was not possible, but their activity was completely suppressed for a period of 18 to 22 hr after the addition of chloramphenicol, as judged by complete lack of glucose fermentation during this period, after which glucose fermentation was resumed, unless a further dose of chloramphenicol was added. All fermentations were done within 12 hr after adding chloramphenicol.

The counting technique. The *in vitro* growth rates were followed by counting protozoa in samples taken at different times. Since the number of protozoa in the cultures was small because of restricted sampling from the calf's rumen, an attempt was made to concentrate the protozoal content of a measured volume into one drop. The initial and final numbers of protozoa were counted in each treatment by withdrawing a 5 ml. sample from a known volume of the culture, under test by using a wide-mouthed graduated pipette. The pipette was left standing vertically for a few minutes until the protozoa contained in the sample sedimented to near the tip of the pipette. A small drop containing all the accumulated protozoa was then pipetted on to a slide and covered with a coverslip (2·3 × 5·1 cm.) so as to spread uniformly under it. Care was taken not to allow the formation of air bubbles. Counting was done at low power having a field area of 1 mm.^{2}. In each slide 30 fields were counted. Three slides were prepared from each sample. This counting technique was simple and gave reproducible results having a coefficient of variability of 6·94 % (ranging from 0·7 to 14 %). The slides were prepared in such a way as to have no more than 15–20 protozoa/field.

Metabolic activity. Washed protozoa in chloramphenicol + CO_2 + buffer were distributed in 10 ml. samples into test-tubes containing a previously weighed amount of substrate. In all incubations the final substrate concentration was 0·20 %. With each run an identical sample of protozoa-free supernatant fluid from the washed suspension was tested with a similar weight of substrate; this latter tube served as a control for detecting bacterial activity (Williams, Davis, Doetsch & Gutierrez, 1961). Bacterial numbers were greatly decreased by the washing used and by the chloramphenicol; this confirms the findings of Coleman (1962, 1967).

All incubations were done at 40° and lasted for 6 hr with soluble sugars and 12 hr with polysaccharides and proteins. After saturation with CO_2 the tubes were stoppered tightly with rubber bungs. After each run the viability of the protozoa was examined microscopically on a warm slide. At the end of each incubation period, the end pro-

ducts in the supernatant fluid were determined and expressed as end product/mg. protozoal-N/hr.

The mono- and di-saccharides were of AR grade (British Drug Houses Ltd.); the polysaccharides were given by Dr Abou Akkada; amino acids, urea, ammonium sulphate and proteins were of GR grade (Merck).

The preparation of cell-free extracts. Cell-free extracts of protozoa were prepared according to Abou Akkada, Eadie & Howard (1963) and kept under toluene in the refrigerator. It was checked for absence of bacteria by culturing in Coleman's media a, b, and c (1962) and found free.

Enzyme activities of cell-free extracts. One ml. citrate phosphate buffer, 0·05 g. substrate in 0·5 ml. buffer solution and 0·2 ml. of cell-free extract were incubated for 1 hr to test for enzyme activities. Incubations were made at different pH values to find the optimum pH values.

Methods of analysis. Reducing sugars were estimated according to Somogyi (1945) with copper sulphate and Nelson's (1944) arsenomolybdate reagents. Sugars were identified on paper chromatograms according to the method of Trevelyan, Procter & Harrison (1950). R_F values were determined for all mono- or di-saccharides used or expected to result from hydrolysis of polysaccharides.

Organic acids were separated for individual determination on silica gel columns according to Bulen, Warner & Burrell (1952) as modified by El-Shazly, Abou Akkada & Naga (1963).

Proteolytic activity was followed colorimetrically by using the ninhydrin reagent (Hawk, Oser & Summerson, 1953). A standard curve was plotted showing the relation between ninhydrin readings and nitrogen concentration of an amino acid mixture (casein acid-hydrolysate). Total N was determined by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943).

Urea was estimated by using soybean meal (British Drug Houses Ltd.) as a source of urease. Ammonia was measured by the microdiffusion technique (Conway, 1957).

Analysis of the protozoa. An acetone-dried mass of washed protozoa was used for determination of the cell composition. Protein ($N \times 6{\cdot}25$) was determined by the micro-Kjeldahl method. Lipids were extracted exhaustively with light petroleum (40°–60°). For the determination of the carbohydrate fraction, proteins and polysaccharides were first dissolved in 30% NaOH (Hungate, 1963); the cellulose and non-cellulose fractions were determined in the alcohol precipitate according to Abou Akkada *et al.* (1963) and Abou Akkada & Howard (1960), respectively. The total sugar content was determined by the anthrone technique (Colvin, Attebery & Ivy, 1961). Glucose was estimated by the specific technique of Hyvariner & Nikkila (1962); this value was subtracted from the anthrone value to obtain the non-glucose sugar fraction.

The amino acid pattern of the protozoal protein was examined by using two-dimensional paper chromatography in phenol–0·3% NH_3 and *n*-butanol + acetic acid + water (40 + 10 + 50 by vol.). Acid hydrolysis was done in sealed tubes with a 6 N-HCl for 22–24 hr. Alkaline hydrolysis was done by boiling in saturated $Ba(OH)_2$ solution for 2 hr.

RESULTS AND DISCUSSION

Eudiplodinium medium (Awerinzew & Mutafowa, 1914) was photographed and described by Hungate (1966) as 'one of the largest rumen protozoa...its macronucleus is depressed at two points on the dorsal side adjacent to the contractile vacuoles (Rees, 1931), it has two skeletal plates'. These morphological characteristics are typical of the species studied in the present work. A photograph of an iodine-stained cell is shown in Pl. 1, fig. 1. Since it was difficult to obtain a clear photograph of the nucleus, the haematoxylin (Heidenhain) stain was applied to a specimen according to Gurr (1953); the stained nuclei are shown in Pl. 1, fig. 2.

The proximate cell composition of Eudiplodinium medium

The proximate analysis of a mass of protozoa of *E. medium* showed the following composition (% of dry matter): crude protein, 42·3; carbohydrates, 49·1; light petroleum extract, 7·8; ash 0·8. The protein content of this organism may appear somewhat high when compared with that of a mixed protozoal population (McNaught, Owen, Henry & Kon, 1954). The authors ascribed the low protein content of their mixed protozoal preparation to its high content of stored polysaccharides; their protozoa had been separated from rumen samples obtained 2 hr after feeding. In the present study, rumen samples were obtained before the morning ration, about 15 hr after the last meal.

The carbohydrate fraction of *E. medium* gave on hydrolysis 60·4% glucose: the remaining 39·6 might have included pentoses from the nucleic acids.

The amino acid pattern of the protein fraction of this organism showed that glutamic and aspartic acids were predominant. The quantitative amino acid analyses of Weller (1957) and Purser & Buechler (1966) showed these two acids to account for about 25% of the protozoal protein. The chromatograms from the acid and alkaline hydrolyses of some organisms showed the presence of all the essential amino acids. Tryptophan was not detected on the chromatograms.

Three suspensions containing about 2600, 600 and 300 of Eudiplodinium organisms/ml. were found to contain 8·71, 2·04 and 0·98 mg. protein/ml. respectively; indicating that the protozoa contained $3{\cdot}35 \pm 0{\cdot}003$ μg. protein/organism.

Growth rates of Eudiplodinium medium

Hungate (1942) obtained a culture containing over 1000 organisms of *Eudiplodinium neglectum*/ml. at 0·08% (w/v) feed concentration; increasing the substrate concentration favoured protozoal growth but the culture medium needed more frequent renewal. Cultures containing fewer protozoa and the same substrate concentration did not survive; it was suggested that this was because of the presence of toxic metabolic products. In the present cultures of *E. medium* the number of organisms never exceeded 50 to 100/ml. with a feed concentration (barley hay) of 0·08% (w/v). Even with as few protozoa as 5 to 10/ml. there was good growth, but only when the feed concentration did not exceed 0·23 mg./protozoa.

Table 1 shows the relationship between substrate concentration (barley hay) in mg./protozoa and the growth of *E. medium*. A concentration of 0·23 mg. feed/protozoon was most favourable for growth. The initial number of protozoa was doubled within 2 hr, as compared with 14 and 7 hr for the concentration of 0·08 and 0·16 mg.

feed/protozoa, respectively. Higher concentrations (0·32 mg./protozoa) caused rupture of the organisms as observed microscopically. It is possible that the higher substrate concentrations per *E. neglectum* was the reason for their non-survival in the cultures of lower population densities prepared by Hungate (1942). On the other hand the longer generation time (48 hr) reported by Hungate (1942) was probably due to the much lower concentration of feed/protozoon (0·0007 mg.) used in his culture. The different generation times reported for different protozoal species (Gutierrez, 1955, 1959; Warner, 1962; Coleman, 1962; Mah, 1962) may have reflected limitations due to substrate concentration/organism rather than a character of the species.

Table 1. *The relationship between substrate concentration (barley hay in Hungate medium) (mg./protozoon) and the concentration of Eudiplodinium medium (organisms/ml.)*

	Substrate concentration (mg./protozoon)			
Time from start (hr)	0·08	0·16	0·23	0·32
	Protozoa (no./ml.)*			
0	10·0	5·0	4·4	2·5
1	10·0	4·8	4·7	3·3
2	11·4	5·9	9·0	2·8
3	12·2	7·6	13·9	2·5
4	13 0	8·4	15·7	—
5	13·8	9·0	17·9	—
6	14·1	9·6	20·6	—
7	14·9	9·9	20·7	1·8
11	18·3	5·7	11·2	0·7
14	20·2	—	—	—

* Averages of three determinations (see text).

Table 2. *Eudiplodinium medium: the exhaustion of stored polysaccharides of protozoa in presence of glucose*

	Glucose in medium		Protozoal polysaccharides	
Time (hr)	μg./ml.	% of initial	μg./ml.	% of initial
0	179·0	100·0	260·0	100·0
4	184·0	102·8	182·5	70·0
7	193·0	108·0	135·0	52·0

The metabolic activities of Eudiplodinium medium

Eudiplodinium medium did not utilize glucose, fructose, mannose, galactose, xylose, arabinose, galacuronate, maltose, sucrose, lactose, cellobiose or xylobiose. The protozoa were alive and showed active movement at the end of the incubation period. It was observed that the sugar concentration in the supernatant fluid after incubation had increased; this must have come from stored polysaccharide in the organisms (Table 2). The protozoa became transparent after 6 hr incubation with the soluble sugars; this is a sign of exhaustion of reserve material.

Eudiplodinium medium did not hydrolyse pectin, pectate, inulin, levan. Di- and poly-saccharides were run on paper chromatograms before and after incubation with *E. medium*; no new spots were observed after incubation. The ability of *E. medium* to engulf cellulose particles was observed microscopically, but no apparent utilization

could be demonstrated. No sugars were released into the medium above that of the endogenous values, because of cellulose ingestion at 12 hr intervals. Incubations with cellulose for longer periods resulted in death of the organisms. Volatile fatty acid yield was somewhat higher (0·079 μ-equiv./mg. protozoal-N/hr) in the cellulose incubations than in the controls. This might have been due to increased endogenous metabolism in the process of ingestion or to the presence of impurities (hemicellulose) in the cellulose preparation. Westphal (1934) observed the same species to ingest cellulose with no resulting deposition of polysaccharide reserves.

Table 3. *Eudiplodinium medium: the soluble end-products of different polysaccharides fermented and the proportions (%) of volatile fatty acids (VFA) produced, expressed as end-product/mg. protozoal-N/hr*

Polysaccharide	Reducing sugar produced above endogenous (μg./mg. N/hr)	Total VFA produced above endogenous (μ-equiv./mg. N/hr)	Lactic acid* produced above endogenous (μ-equiv./mg. N/hr)	Proportions of VFA			
				Formic acid	Acetic acid	Propionic acid	Butyric acid
Rice starch	0·22 ± 0·062	0·43 ± 0·031	0·06	24·53	42·72	4·27	28·48
				25·75	43·34	3·67	27·24
Amylose	0·58 ± 0·081	0·38 ± 0·040	—†	—	—	—	—
Amylopectin	0·96 ± 0·010	0·26 ± 0·040	—	—	—	—	—
Hemicellulose‡	0·64 ± 0·007	0·34 ± 0·036	0·05	18·41	54·74	3·48	23·37
				16·20	59·42	3·33	21·05
Xylan	1·65 ± 0·097	0·02 ± 0·001	0·07	33·75	46·22	3·52	15·51
				32·37	45·96	3·74	17·93
Araban	0·06 ± 0·003	0·34 ± 0·079	—	—	—	—	—
Cellulose	0·00	0·08 ± 0·016	—	—	—	—	—
Endogenous	0·01 ± 0·004	0·23 ± 0·060	0·05	12·85	57·66	4·29	25·20
				11·12	56·67	4·25	27·20

* = one determination. † = not determined. ‡ = purified bran cellulose.

Table 3 gives the products of metabolism of rice starch, amylose, amylopectin, hemicellulose, xylan, araban and cellulose. Starch and hemicellulose are the major sources of energy for this organism. Glucose and maltose were detected on chromatograms prepared from starch, amylose and amylopectin incubations. Xylose, arabinose or both were detected on paper chromatograms prepared from xylan and hemicellulose fermentations respectively.

Eudiplodinium medium is like other protozoa in the general pattern of end products, producing traces of propionic acid and much acetic and butyric acids (Howard, 1963; Hungate, 1966). It also produces relatively large amounts of formic acid which seem to exceed that produced by any other rumen protozoa. Holotrichs do not produce formic acid (Heald & Oxford, 1953; Gutierrez, 1955; Howard, 1959); *Epidinium ecaudatum* (Gutierrez & Davis, 1962) and *Entodinium caudatum* (Abou Akkada & Howard, 1960) produce little formic acid. Succinic acid was not found among the fermentation products of *E. medium* and only a little lactic acid (Table 3).

Xylan was hydrolysed mainly to xylose which was not further degraded appreciably to volatile fatty acids. This could be an example of the phenomenon of hydrolysis of a polymer without utilization of the products which was reported by Abou Akkada & Howard (1961), who found that holotrichs hydrolysed pectic substances without utilizing the hydrolysis products. Dehority (1965) found that three strains of rumen

cellulolytic bacteria degraded and utilized xylan, while five other organisms, particularly *Bacteroides succinogenes*, degraded it. The hydrolysis of xylan by *E. medium* may have significance for the growth of rumen bacteria. Xylose favours protein synthesis (Hendrickx & Martin, 1963), an effect in this respect only surpassed by arabinose. In the present work xylose was not fermented and accumulated, arabinose (or perhaps the biose, as arabinose given externally is apparently not fermented) is fermented as fast as it is formed by hydrolysis of araban and does not accumulate.

Nitrogen metabolism

Urea and ammonium sulphate, in concentrations similar to that of artificial saliva (McDougall, 1948), were not utilized by *E. medium* even in the presence of amylopectin (0·2%). A mixture of 20 amino acids (in concentration as recommended by Quinn, Burroughs & Christiansen, 1962) was not taken up or catabolized by this organism; no ammonia was produced when the organisms were incubated with the amino acid mixture for 6 hr. Abou Akkada & Howard (1962) did not detect any utilization or degradation of Amino acids by *E. caudatum* by using similar techniques. Coleman & Hall (1966) and Coleman (1967), using ^{14}C-labelled amino acids, provided evidence of a slow uptake of amino acids by *E. caudatum*; some volatile fatty acids were found but not ammonia.

Table 4. *Eudiplodinium medium: the end-products of different proteins with and without energy source (amylopectin)*

Protein substrate	NH_3 produced above the endogenous (μg. NH_3-N/mg. protein-N/hr)	Amino acids produced above endogenous (μg. amino-N/mg. protein-N/hr)
Endogenous	0·48	19·62
Endogenous + amylopectin	—	6·65
Casein alone	—	26·00
Casein + amylopectin	—	45·00
Ground casein + amylopectin	—	14·32
Gluten + amylopectin	—	17·00
Ground gluten + amylopectin	—	11·09
Gelatin alone	—	39·21
Gelatin + amylopectin	—	58·36

Eudiplodinium medium produced ammonia and amino acids as end-products of endogenous N metabolism; the presence of amylopectin increased metabolic activity as indicated by higher amino acid production (Table 4). The addition of cysteine HCl (2%) favoured proteolysis and was used in all experiments. Gelatin showed the highest rate of hydrolysis followed by casein. Gluten was the least hydrolysable (Table 4). It appeared that the rate of protein breakdown by *E. medium* ran parallel with the solubility of the protein. The addition of amylopectin to the protein increased proteolysis significantly. However, the proteolytic power of *E. medium* was much lower than that of *E. caudatum* (Abou Akkada & Howard, 1962). When casein and gluten were finely ground, proteolysis was considerably decreased (Table 4).

Activity of cell-free extracts of Eudiplodinium medium

Cell-free extracts of holotrichs were shown to exhibit activities corresponding to those of the whole living protozoa (Howard, 1959). Howard (1963) reported that the holotrich outer membrane was permeable, while that of oligotrichs was not. The cell-free extracts of oligotrichs seem to lack this parallelism in activity with the whole protozoa. Sugden (1953) and Howard (1963) reported that oligotrichs fermented only the insoluble particulated polysaccharides, and did not utilize soluble carbohydrates. The cell-free extracts of oligotrichs exhibited enzymic activities against soluble disaccharides (maltose, sucrose, cellobiose, xylobiose; Abou Akkada & Howard, 1960, 1962; Bailey & Clarke, 1963). On the other hand, there were enzymes detected in living protozoa which were not found in the corresponding cell-free extracts (Bailey & Howard, 1962; Bailey & Clarke, 1963). The cell-free extract of *E. medium* showed no proteinase activity which was observed with the living protozoa. Maltose and xylobiose were hydrolysed by the cell-free extracts of *E. medium* to a very small extent (about 8 %).

Table 5. *Eudiplodinium medium cell-free extract: the specific activity of xylanase in the different ammonium sulphate precipitate fractions of the cell-free extract*

The incubations lasted for 30 min. at 40°. Incubation mixture was: 2 mg. xylan added as 0·2 ml of 1% solution + 0·2 ml. cell-free extract and phosphate + citrate buffer (pH 7).

Crude cell-free extract	Ammonium sulphate (%) precipitate					
	10	20	30	40	50	60
	Xylose producte (μg./mg. N/min.)					
33·4	20·9	19·0	218·0	634·2	938·3	389·0

Figure 1 shows the relationship between pH value and xylanase, 1–4-α-gluconidase, 1–6-α-glucosidase and arabinase activities, when tested under the same conditions. The activities in the cell-free extract of *E. medium* followed the same trend as the activities of the living protozoa (Table 3). The enzymes concerned with hemicellulose degradation (xylanase, arabinase) had different pH optima (7·5 and 5·5, respectively). The amylases had an optimum pH about 6.

Partial purification of the xylanase was effected by using different concentrations of saturated ammonium sulphate. Table 5 shows xylanase specific activity (μg. xylose produced/μg. N/min.) of the crude cell-free extract and in the different ammonium sulphate precipitated fractions. The highest specific activity was found in the 50% ammonium sulphate precipitate which showed a 28·7-fold purification. Optimum pH values and temperature values obtained with the crude preparation were similar to those obtained with the purified preparation (Fig. 1, 2).

Xylanase activity of rumen liquor (Sorensen, 1955), rumen bacteria (Howard, Jones & Purdom, 1960) and of *Fusarium roseum* (Gascoigne & Gascoigne, 1960) have been studied; their respective pH values were found to be 6·3, 5·6 and 6·3. Three attempts were made to determine Michaelis constant of the enzyme xylanase from *E. medium*. Solubilized xylan (treated with hot 0·1 N-HCl) was used; the results were not reproducible. Howard *et al.* (1960) found that xylanase activity of rumen bacteria

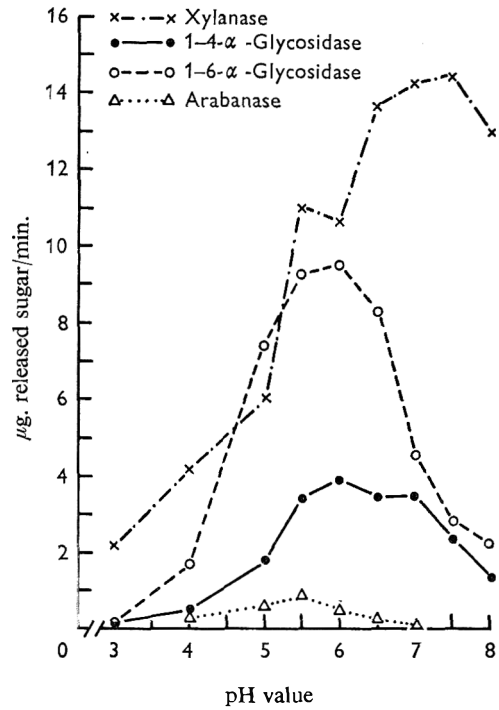

Fig. 1. The pH–activity relationship of enzymes in cell-free extract of *Eudiplodinium medium*. Incubations at 40° for 1 hr. Incubation mixture: 2 mg. substrate added as in 0·2 ml. cell-free extract (crude); 0·6 ml. phosphate+citrate buffer of different pH values.

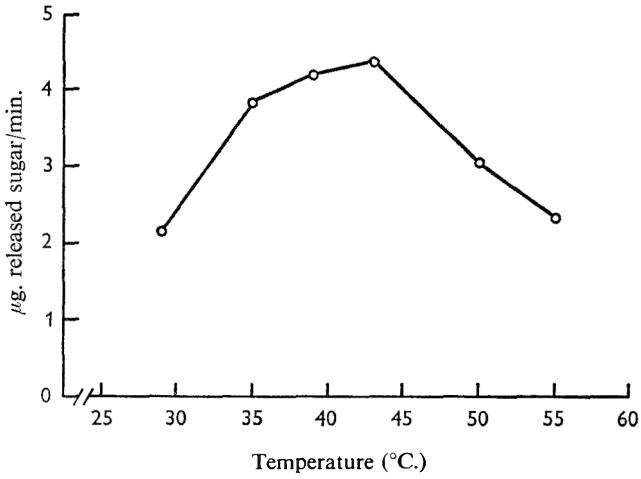

Fig. 2. *Eudiplodinium medium* cell-free extract. The temperature-activity relationship of the enzyme xylanase. Incubations for 1 hr at different temperatures. Incubation mixtures consisted of: 2 mg. xylan added as 0·2 ml. of a 1% solution; 0·2 ml. cell-free extract; 0·6 ml. phosphate+citrate buffer (pH 7).

decreased sharply as the degree of polymerization of the xylan was decreased, and reported that 'the reducing power of the enzyme + pentosan mixture became constant at a value suggesting that the pentosan had been broken down to oligosaccharides with degree of polymerization of about 12'.

In the present work when the prepared soluble xylan fraction was completely hydrolysed, it had a reducing power 13 times as great as before hydrolysis, suggesting a degree of polymerization of 13 units for the solubilized xylan fraction. From this point of view, the short-chain xylan preparation was not expected to respond as native xylan to the enzyme action. The enzyme action on this substrate ceased on hydrolysing 64% of its structure.

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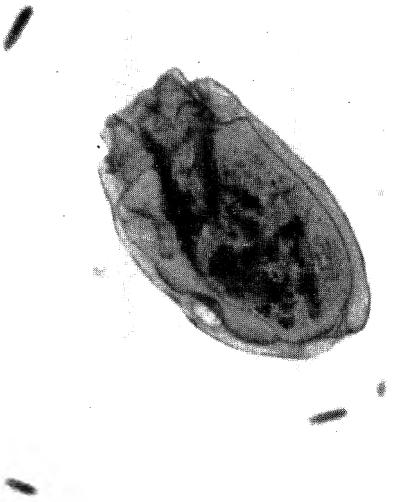

Fig. 1

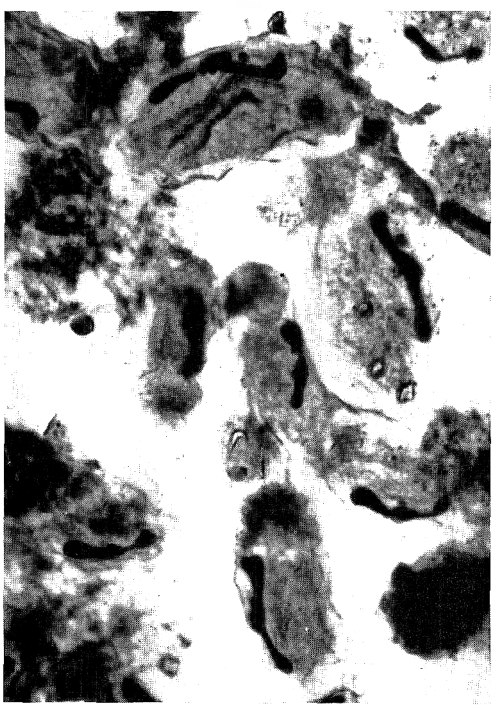

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. *Eudiplodinium medium* from the rumen of buffalo stained with iodine. × 160.

Fig. 2. *Eudiplodinium medium* from the rumen of buffalo. Stained with haematoxylin to show the macronucleus. × 99.

J. gen. Microbiol. (1968), **53**, 317–320
With 1 *plate*
Printed in Great Britain

Amorphosporangium (Actinoplanaceae): Report of Motility and Additional Characters

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(*Accepted for publication* 17 *April* 1968)

SUMMARY

Motility and flagellated sporangiospores are reported for the first time for the type culture (253) of *Amorphosporangium auranticolor*; microconidia on the substrate mycelia are also reported. Otherwise, the morphological description by Couch (1963) remains unchanged. Tests for utilization of 21 carbon sources, gelatin, starch, casein, tyrosine, and cellulose acetate, and for nitrate reduction have been made and the results are reported.

INTRODUCTION

The genus *Amorphosporangium* was described by Couch (1963) as having non-motile sporangiospores. In the present study of *Amorphosporangium auranticolor* (type culture 253), this member of the Actinoplanaceae has been found to have flagellated and motile spores. A more complete characterization of this culture, both physiologically and morphologically, is given.

METHODS

The stock culture of *Amorphosporangium auranticolor* 253, which has been in culture since 1955 on Czapek and peptone Czapek agars (both with 3 %, w/v, sucrose), lost the ability to produce sporangia sometime before 1961 (Couch, 1963). Therefore a culture freeze-dried in 1958 was used.

Inoculum. Peptone Czapek agar plates were covered with a cellulose acetate strip and inoculated. After one week, the growth was scraped off, placed in 30 ml. of sterile distilled water, and blended in a Waring Blendor semimicro cup until a uniform suspension of mycelial material was obtained.

Media. The carbon sources (Table 1) (all but the Na acetate and glycerol obtained from Nutritional Biochemicals Corp., Cleveland, Ohio) were prepared in a 1 % (w/v) concentration in basal Czapek agar ($NaNO_3$, 3·0 g.; K_2HPO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; Difco agar, 15·0 g.; 1000 ml. H_2O), the basal salts mixture prepared by Fisher Scientific special order no. 8548. These agars, nutrient gelatin (peptone, 5·0 g.; beef extract, 3·0 g.; gelatin, 100·0 g.; 1000 ml. H_2O), and nitrate broth (peptone, 10·0 g.; $NaNO_3$, 1·0 g.; 1000 ml. H_2O) in 8 ml. portions in Kimax screw-cap test tubes, autoclaved at 121° for 15 min. were sloped. Four tubes of each medium were inoculated with 0·4–0·5 ml. of blended inoculum by using a hypodermic syringe (no. 18 needle). The inoculum was spread over the surface. Pour plates of starch (soluble starch, 10·0 g.; yeast extract, 2·5 g.; tryptone, 5·0 g.; agar,

15·0 g.; 1000 ml. H_2O), casein (powdered skim milk, 40·0 g.; agar, 15·0 g.; 1000 ml. H_2O), and tyrosine (peptone, 5·0 g.; beef extract, 3·0 g.; L(−) tyrosine, 5·0 g.; agar, 15·0 g.; 1000 ml. H_2O) agars were prepared. Several drops of inoculum were placed on one side of the cooled agar and the plates tilted, allowing the inoculum to run across the centre of the plate in a line. Plates prepared as those for growing the inoculum material were used for the cellulose acetate breakdown. Three per cent sucrose Czapek agar plates were also inoculated to test the culture for sporangial production on this medium.

Table 1. *Amorphosporangium auranticolor growth on various single carbon sources*

Carbon source	Relative growth	Carbon source	Relative growth
L(+) Arabinose	++++	d(+) Lactose	++++
d(+) Xylose	+	d(+) Maltose	++++
L(+) Rhamnose	++++	d(+) Cellobiose	++++
Salicin	+++	d(+) Sucrose	++++
i-Inositol	+++	d(+) Raffinose	++++
d(−) Glucose	++++	Amylopectin	++++
d(−) Mannitol	++++	Inulin	+
d(−) Levulose	++++	Na Succinate	++
d(+) Galactose	++++	Na Acetate	+++
Dulcitol	++++	Glycerol	++++
d(+) Sorbitol	++++		

++++ = excellent growth; +++ = fair growth; ++ and + = poor growth.

All cultures were incubated at room temperature (25 to 27°) for one month before results were recorded.

Electron microscope preparations. A sporangial suspension was prepared from a 1-month-old sorbitol agar slope culture by adding 5 ml. sterile distilled water to the culture and scraping the surface to loosen and wet the sporangia. After 30 min. the spores were swimming. The preparation was mounted on 200-mesh copper grids by the agar block technique (Sharp, 1953) and shadowed with germanium. Pictures were taken on Kodak Fine Grain Positive 35 mm. film with a Phillips EM 75.

RESULTS

Morphology

Many sporangia on both the Czapek agar plates and the sorbitol agar slopes were irregular in shape (Pl. 1, fig. 1, 4) and similar to those reported by Couch (1963). An electron micrograph of an empty sporangium is shown in Pl. 1, fig. 1 A, illustrating its irregular shape with four or five lobes. The sporangial wall and a portion of the upper sporangiophore had a rough-textured surface, whereas the vegetative hyphae (Pl. 1, fig. 1 B) had smooth walls. Plate 1, fig. 4, illustrates a similar sporangium with a light micrograph. 'Microconidia', which Couch (1963) described on substrate mycelia of other Actinoplanaceae (*Ampullariella campanulata, Actinoplanes utahensis*), were observed (Pl. 1, fig. 5). Couch also illustrated these in his light microscope studies of the genus *Amorphosporangium* (unpublished data, personal communication). The branched coiled arrangement of the spores can be seen in the partially broken-open sporangial

lobe in Pl. 1 fig. 6. Flagellated spores are present on the upper edge of the lobe where the sporangial wall is broken. Plate 1, fig. 2, shows two rod-shaped spores, the lower one displaying a polar tuft of flagella as reported for many other Actinoplanaceae (Higgins, Lechevalier & Lechevalier, 1967; Kane, 1966; Lechevalier & Holbert, 1965). The shape of the spores varied from spherical to rod shaped, and a few curved spores were seen (Pl. 1, fig. 3). The majority, however, were short rods.

Physiology

Table 1 gives the results of growth of *Amorphosporangium auranticolor* 253 on various carbon sources. Cultures with substrates which supported excellent growth (indicated by confluent surface growth which penetrated the agar approximately 1/8 of an inch) also hadorange pigmentation. With glycerol, the culture also produced a brown pigment which diffused into the agar. Fair growth but no orange pigmentation was obtained with salicin, inositol, and Na acetate. There was poor growth (barely visible or visible only with a dissecting microscope) with xylose, inulin, and Na succinate. A few sporangia were produced on dulcitol, whereas the surface of the sorbitol agar was entirely covered with sporangia. These two were the only carbon sources in slopes on which sporangia were formed. Nitrate reduction and tyrosine utilization were negative. Gelatin liquefication, starch and casein hydrolysis, and cellulose acetate breakdown were positive.

DISCUSSION

Although this work has shown *Amorphosporangium auranticolor* to have basically rod-shaped spores with a tuft of polar flagella, as has been reported for the genus *Ampullariella* (Higgins *et al.* 1967; Kane, 1966), it retains the distinction of having these rod-shaped spores arranged in coils within the irregularly lobed sporangium. It remains distinct from the genus *Actinoplanes*, which has round to ovoid spores usually arranged in coils within round or only slightly irregular sporangia.

It is understandable that Couch did not observe motility in Amorphosporangium. In many of the Actinoplanaceae, motility in the same culture will vary from one day to the next for no apparent reason. Also, even slight differences in cultural conditions such as osmotic pressure or age of the culture may affect motility (Higgins, 1967).

With the discovery of motility in *Amorphosporangium auranticolor* only the genus *Streptosporangium* Couch remains non-motile. The genus *Microellobosporia* has been tentatively transferred to the Streptomycetaceae on the basis of cell-wall composition (Lechevalier & Lechevalier, 1967).

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EXPLANATION OF PLATE

Amorphosporangium auranticolor

Fig. 1 A. Empty sporangium with lobes (L) and showing texture of sporangial wall and sporangiophore (SP).

Fig. 1 B. Fragments of vegetative hyphae (VH) and the bases of two sporangiophores, showing the differences in wall textures.

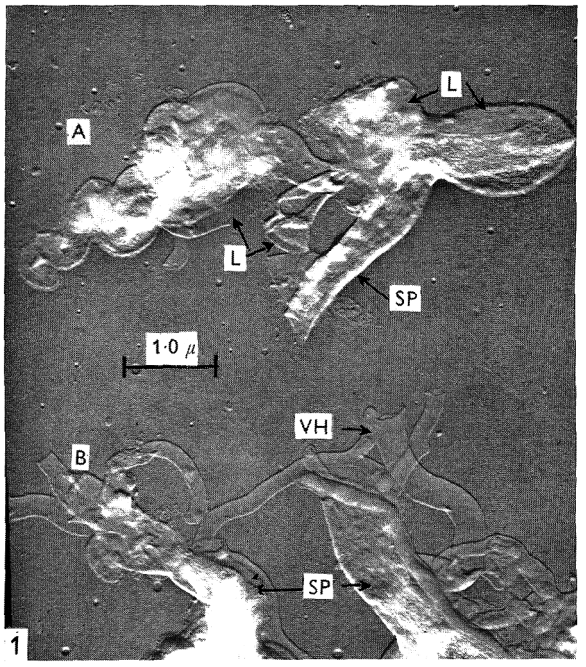
Fig. 2. Two spores, the lower with a tuft of polar flagella.

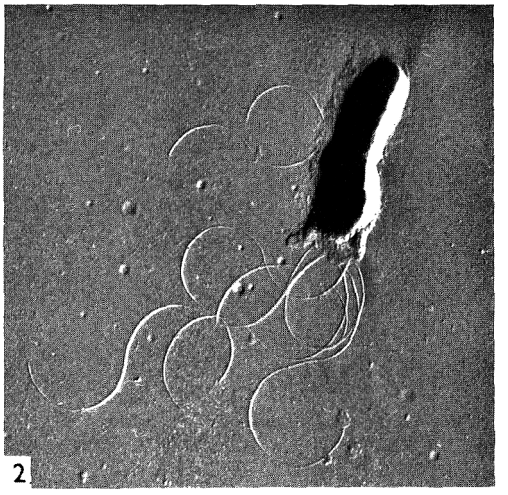
Fig. 3. Curved spore with polar flagella.

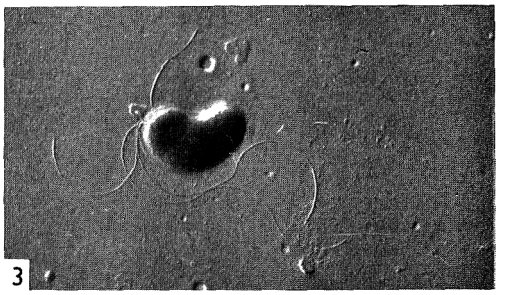
Fig. 4. Light micrograph of lobed sporangium.

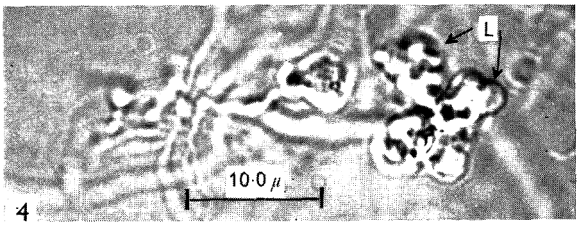
Fig. 5. Hypha with a 'microconidium' (MC).

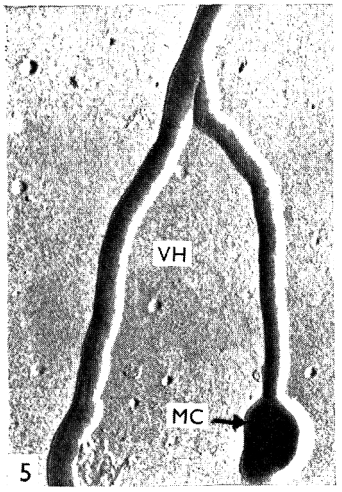
Fig. 6. Lobe of sporangium with spores showing coiled and branching (B) pattern of spores. One micron marker for figs. 2, 3, 5 and 6.

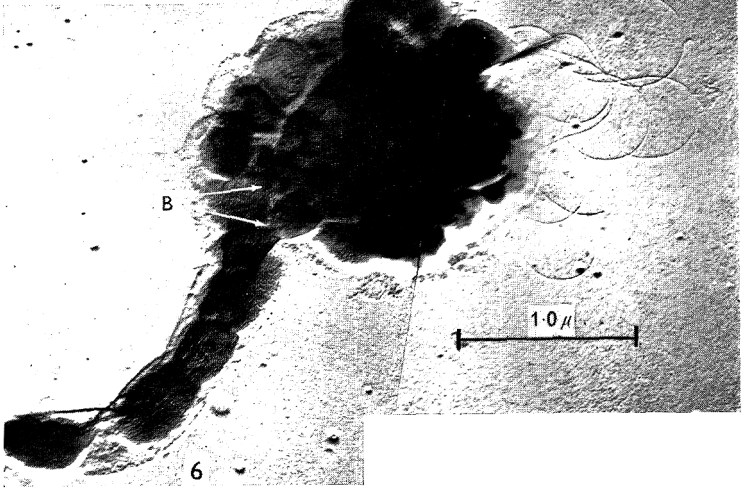












W. K. HANTON

(*Facing p.* 320)

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With 1 *plate*
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The Mating-type Systems of the Myxomycetes *Physarum rigidum* and *P. flavicomum*

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(*Accepted for publication* 18 *April* 1968)

SUMMARY

Three geographical collections (isolates) of myxomycetes originally identified as *Physarum flavicomum* were found to be heterothallic and the clones established from each isolate were divided into two mating types. Previous studies of six isolates of *P. flavicomum* showed that they could be divided into three groups. Mating occurred within groups but not between groups, and within each group there were two or more mating types. When the new isolates were crossed to the original isolates, they conformed to two of these three groups. This study established the existence of multiple alleles at the incompatibility locus in group 3. Mating-type alleles of the F_1 generation of group 3 segregated in a 1 : 1 ratio at meiosis. Isolates belonging to group 1 were also separated from the other two groups on morphological and physiological bases. The morphological description fits that of *P. rigidum* more nearly than *P. flavicomum* and group 1 isolates were placed in the species *P. rigidum*. Groups 2 and 3 were separated genetically and physiologically but morphologically the separation was not distinct. These groups were referred to as *P. flavicomum* varieties 1 and 2.

INTRODUCTION

The life cycle of the myxomycetes (Alexopoulos, 1963) is initiated by the liberation of myxamoebae from the haploid spores. The myxamoebae divide mitotically and when a large population has formed two myxamoebae fuse to form a diploid zygote. Karyokinesis continues but cytokinesis fails to take place resulting in a large multinucleate plasmodium. Under certain environmental conditions fruiting bodies are formed; meiosis takes place with the formation of the haploid spores. In heterothallic myxomycetes the two myxamoebae that fuse to form the zygote must be of opposite mating types and come from different spores.

The list of heterothallic myxomycetes has grown considerably since Dee (1960), with *Physarum polycephalum*, first established the existence of an incompatibility mechanism in this group of organisms. These include *Didymium iridis* (Collins, 1961), *P. pusillum* (Collins, 1962), *Fuligo septica* (C. J. Alexopoulos & M. R. Henney, unpublished data), *P. globuliferum* (M. R. Henney, unpublished data), *Comatricha laxa* (Wollman, 1966), and *P. flavicomum* (Henney, 1967). It has further been shown that both homothallic and heterothallic isolates occur in the same species and that these isolates are indistinguishable on morphological grounds. The term 'isolate' designates a specimen collected at a specific geographical location at a specific time. Collins (1965) reported a homothallic isolate of *D. iridis*. C. J. Alexopoulos & M. R. Henney (unpub-

lished data) found three of five isolates of *P. pusillum* to be heterothallic and the other two to be homothallic. They found similar results while working with *F. septica*, four of seven isolates were heterothallic and three were homothallic. This could explain the contradictory results obtained by von Stosch (1935), who reported *D. nigripes* to be heterothallic, and Kerr & Sussman (1958), who reported the same organism to be homothallic.

The existence of multiple alleles at the incompatibility locus has been reported in heterothallic myxomycetes. This has been established for *Didymium iridis* (Collins 1963), *Fuligo septica* (C. J. Alexopoulos & M. R. Henney, unpublished data), *Physarum polycephalum* (Dee, 1966), and *P. flavicomum* (Henney, 1967). Six isolates of the latter (each consisting of two mating types) could be divided into three groups based on their ability to cross with one another. Members of the first group crossed with each other in such a way as to indicate the existence of six different mating types. Members of the second group again crossed with other members of their group, indicating four mating types. The sixth isolate could be divided into two mating types that would not cross with the remaining five isolates. These six isolates could also be divided into three corresponding morphological groups based on spore size, shape of sporangium and stalk length. Further studies of three more isolates originally identified as *P. flavicomum* have been conducted and compared to the six original isolates (Henney, 1967).

METHODS

Cultures. The three new geographical collections (isolates) were all found in Texas and are referred to as TEXAS III, TEXAS IV and TEXAS V. Isolates TEXAS III and TEXAS IV were collected by the authors in Bastrop State Park near Austin, Texas, November 1965. These isolates were collected at the same time and within a few yards of one another. The TEXAS V isolate originated in a moist chamber composed of debris collected near San Antonio, Texas, 1964, and was kindly supplied by Dr Constance Wollman. The sources of the original six isolates, TEXAS I, PHILIPPINE I, COSTA RICA, PHILIPPINE II, AFRICA and TEXAS II, are given in an earlier paper (Henney, 1967).

Clonal establishment. Clones were established by distributing a suspension of spores over the surface of a plate of half-strength corn meal agar containing 8·5 g. Difco (Detroit, Michigan, U.S.A.) corn meal agar, 12·5 g. Difco plain agar, and 1000 ml. distilled water. Under 120 magnification a single spore was isolated by cutting-out, with a fine sterile entomological needle, a small block of agar upon which it rested. The agar block bearing the spore was transferred to a 60 × 20 mm. sterile Petri dish containing 8 ml. half-strength corn meal agar to which was added 0·5 ml. of a dilute suspension of *Aerobacter aerogenes* or *Escherichia coli*. The single-spore cultures were incubated at 25° and were examined after 2 and 4 weeks. By this time there was a large myxamoebal population in all plates where germination had occurred. Each population originated from a single spore and is referred to as a clone.

Axenic clones. Axenic and monoxenic clones were established by placing 3 or 4 drops of a myxamoebal suspension near the edge of a half-strength corn meal agar plate and tilting the plate to allow the suspension to run across the agar. The plates were incubated at 25° for several days. The contaminating bacteria formed colonies all along the line of inoculation. By the end of one week the myxamoebae had multiplied and large numbers of them had crawled away from the line of inoculation and

from the bacteria. A small agar block containing 20–30 myxamoebae was cut out and transferred to a second half-strength corn meal agar plate. Axenic and monoxenic clones were prepared by adding 0·5 ml. of a formalin-killed, or living bacterial suspension, respectively, to the block of agar containing the myxamoebae. To test the purity, five other agar blocks were cut out immediately adjacent to the original block. Each block was placed in a different bacteriological medium. The media used included glucose broth, yeast extract broth, AC broth, Bristol's solution, and thioglycollate broth (Henney, 1966). Small agar blocks were cut out from axenic cultures and placed in the above media. The media were all incubated at 25° and 37° for 30 days. When bacterial growth developed, as indicated by turbidity, in any of the five test media, the entire experiment was discarded and the whole procedure repeated.

Crosses. Crosses were made by inoculating 5 drops of a myxamoebal suspension, by using a sterile Pasteur pipette, from each of two different clones on to a 60 × 20 mm. Petri dish containing 8 ml. of half-strength corn meal agar. A living bacterial suspension, 0·25 ml., was added to each cross. All crosses were made in duplicate. In addition, duplicate cultures of the individual clones were always used as controls. Crosses were examined after 1 week and 1 month and scored + or − for plasmodial formation. Plasmodial formation was always the criterion used for compatibility.

RESULTS

Intra-isolate crosses

Thirty clones were established, as described in methods, from the TEXAS III isolate and ten of these, selected at random, were crossed in all possible combinations. The results indicated the clones were of two mating types. The remaining 20 clones were crossed to two tester clones of the two mating types selected on the basis of the first results. Eleven of the clones formed plasmodia when crossed to one of the tester clones but not with the 2nd. The remaining nine clones formed plasmodia when crossed to the 2nd tester clone but did not form plasmodia when crossed to tester clone number 1. Similar results were obtained with the TEXAS IV and TEXAS V isolates.

Inter-isolate crosses

Inter-isolate crosses were always made by crossing ten clones of each isolate (five of each mating type) with ten clones of every other isolate. The results of inter-isolate crosses of the original six isolates showed that three compatibility groups were formed containing three series of incompatibility alleles. Group 1 consisted of the TEXAS I, PHILIPPINE I and COSTA RICA isolates. Following the example of Collins (1963) the mating types of the different isolates were assigned the allele numbers A 1 and A 2 for the TEXAS I isolate, A 3 and A 4 for the PHILIPPINE I isolate and A 5 and A 6 for the COSTA RICA isolate. Group 2 consisted of the PHILIPPINE II and AFRICA isolates. These two isolates crossed among themselves but would not cross with the other four isolates. The two mating types of the PHILIPPINE II isolate were assigned allele numbers B 1 and B 2, and the AFRICA isolate B 3 and B 4. The third group consisted of the single isolate TEXAS II. The TEXAS II isolate would not cross with any of the other isolates. The two mating types of this isolate were assigned the allele numbers C 1 and C 2.

The inter-isolate crosses involving the three new isolates were made to see if they would fit into the existing compatibility groups or form still more groups. The results

indicated the TEXAS V isolate belonged to group 1 and as both mating types of this isolate crossed with all six established alleles, the mating types were allelic to but different from those of the original isolates. These new mating types were assigned allele numbers A 7 and A 8. Previous to this study it was presumed that multiple alleles

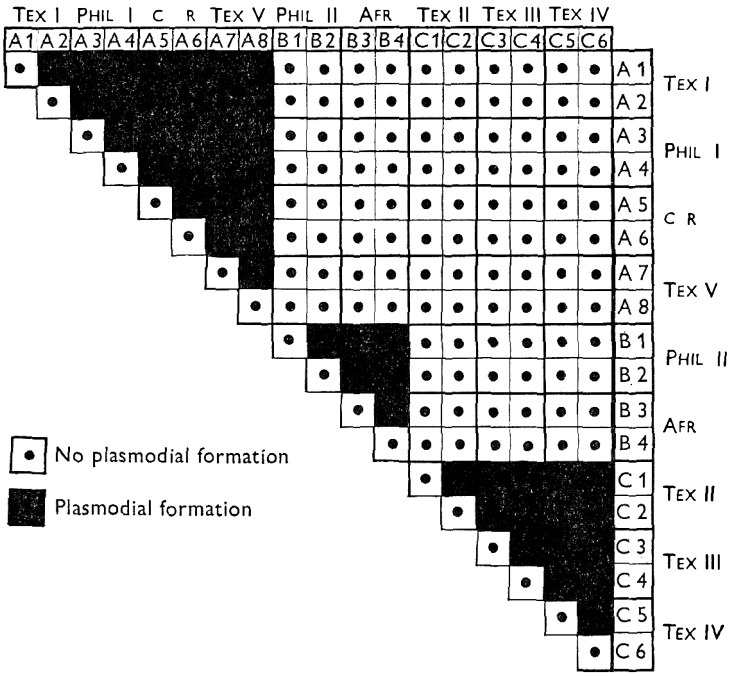

Fig. 1. *Physarum flavicomum*. Results of the intra-isolate, inter-isolate and inter-group crosses of all nine isolates. The isolates are divided into three compatibility groups. Group 1 consists of the TEXAS I, PHILIPPINE I, COSTA RICA and TEXAS V isolates and have been assigned mating-type alleles A 1–A 8. These isolates cross among themselves but not to the other five isolates. Group 2 consists of the PHILIPPINE II and AFRICA isolates and have been assigned mating-type alleles B 1–B 4. The third compatibility group consists of the TEXAS II, TEXAS III and TEXAS IV isolates. These three isolates cross among themselves but not to the other six; they comprise mating-types alleles C 1-C 6.

existed at the incompatibility locus in group 3 as well as the other two groups but this was not definitely established as only a single isolate of this group had been studied. However, when clones of the TEXAS III isolate were crossed to clones of the TEXAS II and TEXAS IV isolates it was found that both mating types crossed with both mating types of the other two isolates, proving the existence of multiple alleles at the incompatibility locus in group 3. It is especially interesting that these two isolates had different mating-type alleles as they were collected at the same time and in very close proximity. The two mating types of the TEXAS III isolate were assigned allele numbers C 3 and C 4. Those of the TEXAS IV isolate were assigned C 5 and C 6. The results of all the inter-isolate crosses are summarized in Fig. 1.

F_1 generations

Ten F_1 clones were established by isolating single spores from sporangia obtained by crossing two compatible clones of each isolate (intra-isolate cross). When these clones were crossed among themselves in all possible combination, in duplicate, they could again be segregated into two mating types that were inherited in a 1 : 1 ratio. The

Table 1. *Ten clones of the F_1 generation of Physarum flavicomum* TEXAS III *crossed in duplicate to tester clones of the two mating types of each of the nine isolates*

Clone numbers	TEX I		PHIL I		CR		TEX V		PHIL II		AFR		TEX II		TEX III		TEX IV	
	A 1	A 2	A 3	A 4	A 5	A 6	A 7	A 8	B 1	B 2	B 3	B 4	C 1	C 2	C 3	C 4	C 5	C 6
F_1S2	o	o	o	o	o	o	o	o	o	o	o	o	×	×	o	×	×	×
F_1S3	o	o	o	o	o	o	o	o	o	o	o	o	×	×	o	×	×	×
F_1S4	o	o	o	o	o	o	o	o	o	o	o	o	×	×	o	×	×	×
F_1S10	o	o	o	o	o	o	o	o	o	o	o	o	×	×	o	×	×	×
F_1S1	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S5	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S6	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S7	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S8	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S9	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×

× = plasmodial formation. o = no plasmodial formation.

Table 2. *Ten clones of the F_1 generation established from plasmodial genotype C 2 × C 4 crossed in duplicate to tester clones of the two mating types of each of the nine isolates*

Clone numbers	TEX I		PHIL I		CR		TEX V		PHIL II		AFR		TEX II		TEX III		TEX IV	
	A 1	A 2	A 3	A 4	A 5	A 6	A 7	A 8	B 1	B 2	B 3	B 4	C 1	C 2	C 3	C 4	C 5	C 6
F_1S3	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S5	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S6	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S8	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S9	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S10	o	o	o	o	o	o	o	o	o	o	o	o	×	o	×	×	×	×
F_1S1	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S2	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S4	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×
F_1S7	o	o	o	o	o	o	o	o	o	o	o	o	×	×	×	o	×	×

× = plasmodial formation. o = no plasmodial formation.

10 F_1 clones were crossed back to each parent clone and also crossed with one tester clone of each mating type of the other eight isolates. Table 1 shows the results of ten F_1 clones established from the TEXAS III isolate in such a cross. In this cross the parent clones are represented by alleles C 3 and C 4. Four of the F_1 clones crossed with parent clone C 4 but not with C 3 indicating these four clones were of the same mating type as parent C 3. The other six F_1 clones crossed with the parent clone C 3 but not with C 4 indicating the remaining six F_1 clones were of mating type C 4. All ten F_1 clones established from the TEXAS III isolate crossed with mating types C 1, C 2, C 5 and C 6 as expected in a multiple allelic system. A similar pattern was obtained for

the TEXAS IV isolate. The F_1 clones of the TEXAS V isolate could be divided into two mating types when crossed back to the two parent clones (A 7, A 8). All F_1 clones crossed with the tester clones of the other three isolates in group 1 but failed to cross with clones of groups 2 and 3.

Inter-isolate crosses within group 3 gave a potential of 12 parental plasmodia. These were C 1 × C 3, C 1 × C 4, C 1 × C 5, C 1 × C 6, C 2 × C 3, C 2 × C 4, C 2 × C 5, C 2 × C 6, C 3 × C 5, C 3 × C 6, C 4 × C 5, C 4 × C 6. F_1 clones were established from sporangia derived from plasmodia of each of the above types. The clones were then crossed back to the parent clones and to tester clones of both mating types of the other eight isolates. The results of one such cross is given in Table 2 where clones C 2 and C 4 were the parental clones. Single-spore isolations were made from sporangia that formed from the resulting plasmodia. The F_1 clones were divided into two mating types, six crossed with one parent clone (C 4) but did not cross with the second parent (C 2). The remaining four clones crossed with parent C 2 but not with parent C 4. All ten F_1 clones crossed with clones of mating type C 1, C 3, C 5 and C 6, but did not cross with any clones belonging to the other two incompatibility series. F_1 clones established from sporangia of the other 11 parental genotypes gave similar results in that the clones could be segregated into the two mating types comprising the parental genotype. None of the F_1 clones of the group 3 isolates would cross with clones established from isolates belonging to groups 1 or 2.

Comparative morphology of the three genetic groups

A comparative morphological study was made of the nine isolates. Some of the morphological characteristics studied were shape of sporangium, length of stalk, and diameter of spores. Preparation of materials was according to Henney (1967). These included sporangia developed under a variety of environmental conditions to exclude the possibility that the variations were environmentally induced. The diameter and width of the sporangia were measured and plotted as a ratio of diameter of sporangium: width of sporangium. The length of the stalks and diameter of spores were also measured.

The study of stalk length divided the six original isolates into three distinct groups (Henney, 1967). Group 1 had short stalks, group 2 had very long slender stalks, while the stalks of group 3 were intermediate. When this morphological character was studied in the three new isolates they, too, could be placed in two of the three groups corresponding to their respective genetic groups (Fig. 2). The TEXAS V isolate, which crossed with group 1, also had short stalks about 0·5 mm. in length, typical of this group. The TEXAS III and TEXAS IV isolates, belonging to genetic group 3, were placed in morphological group 3. The separation from group 2 was not as complete in this case as the stalks were somewhat longer than previously indicated and tended to bridge the gap between groups 2 and 3.

The ratio of diameter of sporangium: width of sporangium again separated the isolates into morphological groups corresponding to their respective genetic groups (Fig. 3). The isolates comprising group 1 were all very lenticular and the diameter of the sporangia about 3 times the thickness. The TEXAS V isolate belonged to this group. Group 3 consisted of a much smaller sporangium and was globose to subglobose with the ratio approaching 1·0 (TEXAS III and TEXAS IV). Group 2 was intermediate, with a

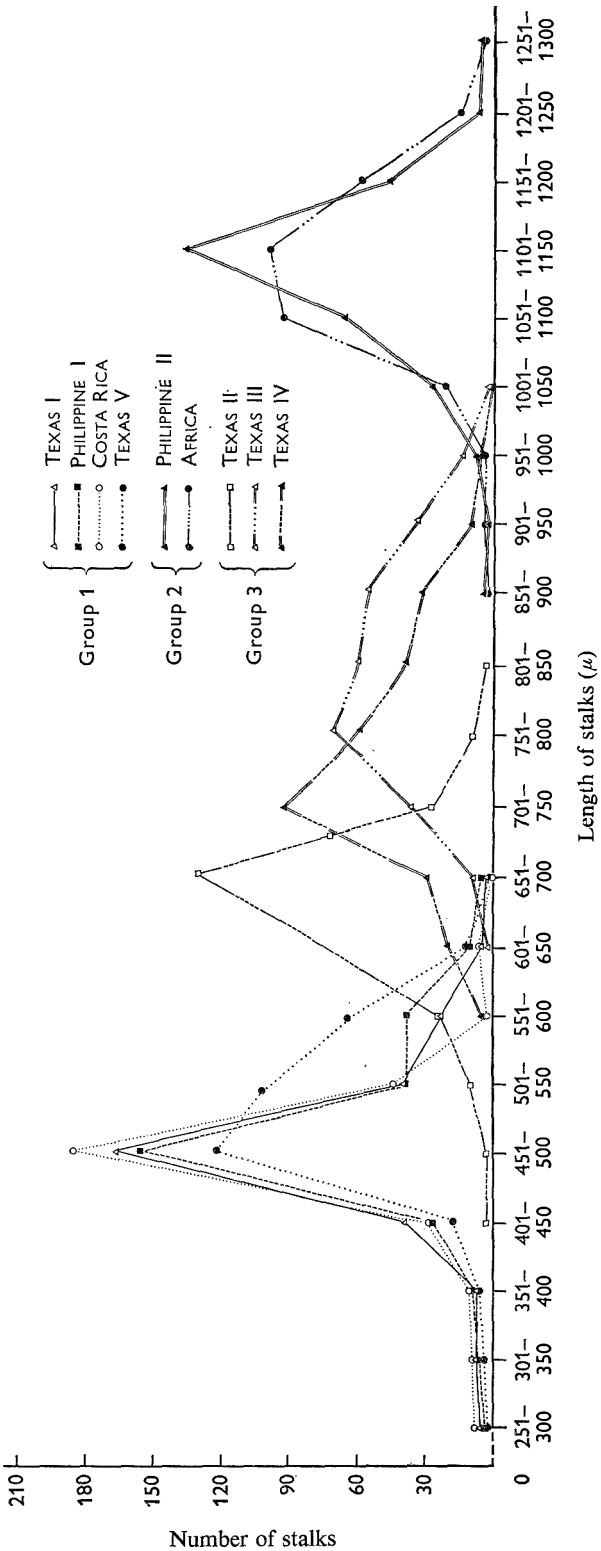

Fig. 2. *Physarum flavicomum*. Comparison of stalk length of the nine isolates. The TEXAS I, PHILIPPINE I, COSTA RICA and TEXAS V isolates comprise one group with an average stalk length of 500 μ. The PHILIPPINE II and AFRICA isolates have a stalk length of 1100 μ and comprise group 2. The third group consists of the TEXAS II, TEXAS III and TEXAS IV isolates with intermediate stalk length.

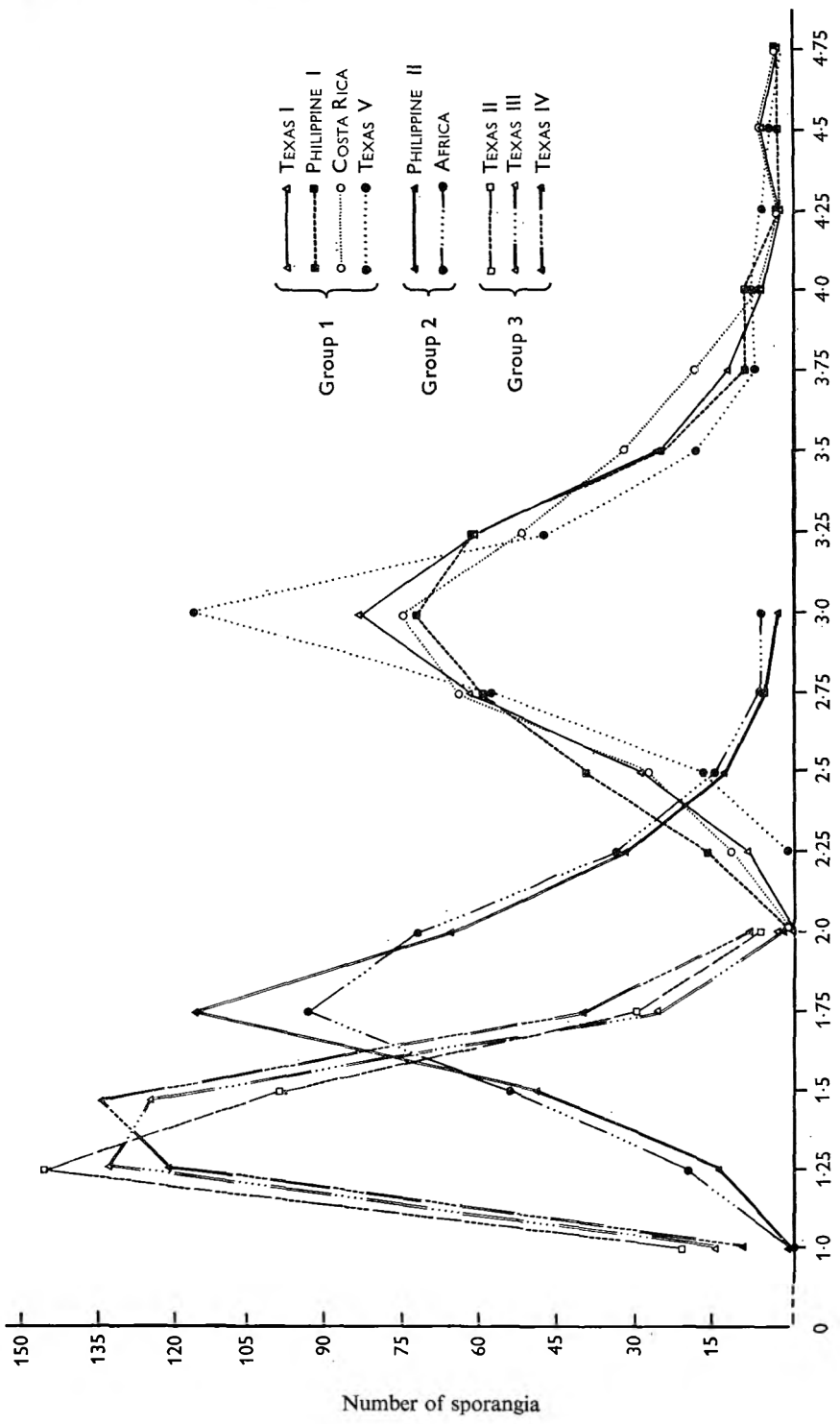

Fig. 3. *Physarum flavicomum*. Comparison of the diameter of sporangium: width of sporangium of the nine isolates. The isolates comprising group 1 have an average ratio of 3·0 as the sporangia are very lenticular. The isolates of group 3 are almost globose, with a ratio approaching 1·0. The two isolates that make up group 2 have a sporangium that is subglobose to lenticular and intermediate between groups 1 and 3.

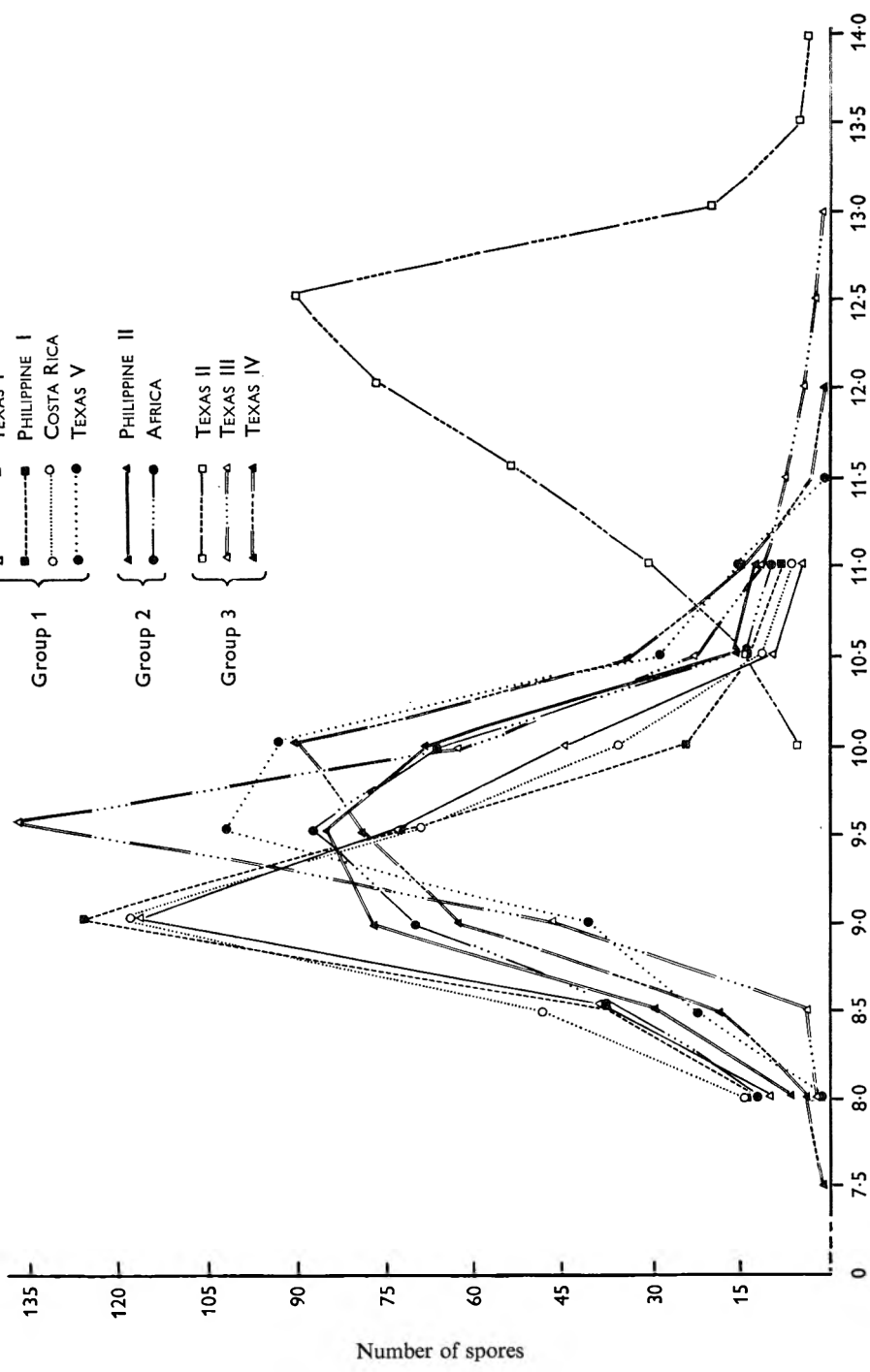

Fig. 4. *Physarum flavicomum*. Comparison of spore size of the nine isolates. Spore size of eight of the isolates was very close with a difference of about 1 μ. The TEXAS II isolate had an average spore size of 12·5 μ. However, this isolate belongs to the same genetic group as the TEXAS III and TEXAS IV isolates with spores that averaged about 9·5 μ in diameter.

somewhat larger sporangium than group 3, and tended to be umbilicate above. Again the two new isolates tended more to combine groups 2 and 3 than to separate them.

The original study indicated the spores of group 3 (this consisted of the single isolate TEXAS II) were much larger than those of groups 1 and 2. However, the comparative study of TEXAS III and TEXAS IV isolates, which belonged to the same genetic group as the TEXAS II isolate, had spores that were indistinguishable from those of groups 1 and 2, indicating spore size is variable within group 3, and this character cannot be used to separate group 3 from the other two groups.

The capillitium of the TEXAS V isolate was characteristic of the group 1 type (Pl. 1, fig. 1). Here the capillitial threads were sparse and started at the base of the sporangium and extended to the top of the peridium with little or no branching. In contrast the capillitia of groups 2 and 3 were very dense with the threads starting at the base of the sporangium and approximately half-way up, branched profusely and formed a dense network toward the peridium. The capillitia of TEXAS III and TEXAS IV were of this type (Pl. 1, fig. 2).

DISCUSSION

Taxonomic implications immediately became evident when it was found that *Physarum flavicomum* could be divided into three compatibility groups based on their mating-type reactions. Using the key of Drs G. W. Martin and C. J. Alexopoulos (personal communication: in preparation) all nine isolates used in this study keyed out to *P. flavicomum*. However, using the older keys of Lister (1925), Macbride & Martin (1934) and Martin (1949), the isolates comprising group 1 keyed out to *P. rigidum* except for the absence of the rigid rod-like tubes which characterize that species. Both species belong to a very closely related group called the '*Physarum viride* complex' consisting of *P. viride*, *P. nutans*, *P. rigidum*, *P. bethelii* and *P. flavicomum*. All are separated by relatively minute differences. The problem becomes even more complex as it has been impossible to grow *P. viride*, *P. nutans* and *P. bethelii* even in crude culture.

As more studies were made it became more apparent that those isolates comprising group 1 were different from those comprising groups 2 and 3. This is true genetically, morphologically and physiologically. The shape of the sporangium in group 1 was large and very lenticular, whereas sporangial shape in groups 2 and 3 tended to be globose to subglobose. The length and type of stalk in group 1 was short and thick as compared to stalks of the other two groups and was dark below from included refuse matter. The stalk of *Physarum flavicomum* was free from all inclusions (Lister, 1925).

The most important difference separating group 1 from groups 2 and 3 was that of capillitium. These differences are pictured in Pl. 1, fig. 1 and 2. Macbride & Martin (1934) stated that the capillitium of *Physarum rigidum* consisted of sparingly branched threads with long orange-yellow nodes, or consisted almost entirely of slender, rod-like tubes enclosing yellow lime granules. The capillitium of isolates in group 1 consisted entirely of sparingly branched threads but the slender, rod-like tubes which characterizes this species were never found. It was because of the absence of these calcareous rods that the group 1 isolates were originally placed in *P. flavicomum*. However, the description of the capillitium of *P. flavicomum* is given as 'Capillitium a close network of hyaline threads with numerous yellow flat expansions at the axils, often persistent and retaining the form of the sporangium after the dispersion of the

spores;...'. Whereas this was an accurate description of the capillitia of groups 2 and 3, it did not describe the capillitium of group 1. Since the type of capillitium is considered to be an important taxonomic characteristic in the classification of the myxomycetes (Alexopoulos, 1967), the inclusion of isolates with such different capillitial types in the same species seemed to be unwarranted. Capillitia of groups 2 and 3 were indistinguishable.

The morphology of the plasmodium has not been used taxonomically to any great extent in the identification of the myxomycetes. Alexopoulos (1960, 1963, 1966) has recently focused attention on this aspect and has described three general types of plasmodia corresponding to the major groups in the myxomycetes. However, little significance has been placed on the differences in plasmodia within the different groups, mainly because of the variability of the plasmodium under different environmental conditions. Recently, isolates from each of the three before-mentioned groups have been put in pure culture on a partially defined medium (Henney & Henney, 1968), thus enabling growth under controlled conditions. The plasmodial characteristics of the different groups have been constant for about 1 year and showed distinct differences in morphology and colour.

Another difference separating the three groups was the time required after mating for plasmodial formation to take place. Plasmodial formation in group 1 occurred 5–6 days after clones of opposite mating types were mixed together. In group 2 the time period for plasmodial formation was nearly 2 weeks, whereas 3–4 days was sufficient for this process with the isolates comprising group 3.

It has been decided that the isolates comprising group 1 are much more appropriately placed in *Physarum rigidum* than in *P. flavicomum* because (1) they belonged to different genetic groups, (2) there were differences in the morphology of the sporophores, (3) there were differences in the morphology of the plasmodia in pure culture under controlled environent, and (4) there were physiological differences of time required for plasmodial formation. All these are evidence that these isolates belong to different species. This same reasoning can be applied to those isolates belonging to groups 2 and 3. They, too, belonged to different genetic groups, there were also some differences in the morphology of the sporophore, the plasmodia in pure culture were quite different and the required time for mating was significantly longer for group 2. In this case, however, the morphological differences were much more subtle and the morphological characteristics of the new isolates used in this study tended to bridge the gaps instead of separating them into distinct entities. It is very possible that groups 2 and 3 also belong to different species. After many more isolates are studied a pattern may emerge by which these two groups can readily be identified, but presently we are retaining both groups in *P. flavicomum* as varieties: group 2 = *P. flavicomum* variety 1, group 3 = *P. flavicomum* variety 2.

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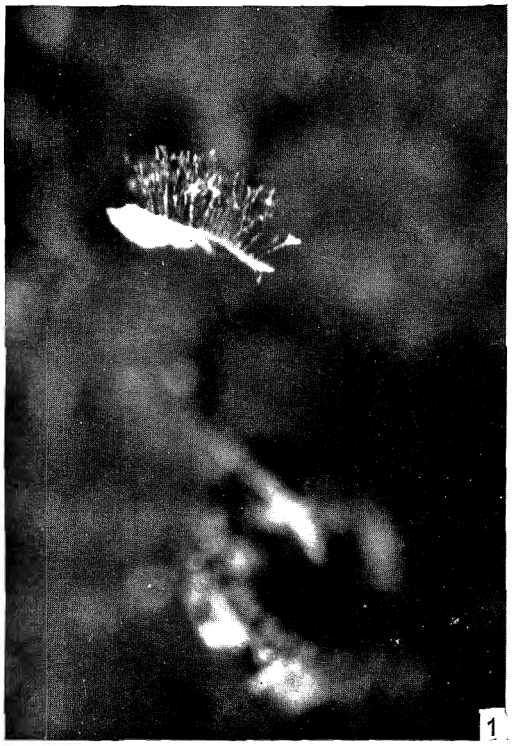
EXPLANATION OF PLATE

Fig. 1. Capillitium of *Physarum rigidum.* The threads start at the base of the sporangium and go to the peridium with little or no branching. The magnification is about 80 ×.

Fig. 2. Capillitium of *Physarum flavicomum.* The threads start at the base of the peridium but soon form a profusely branching network. The magnification is about 90 ×.



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Nutritional Requirements for the Growth in Pure Culture of the Myxomycete *Physarum rigidum* and Related Species

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SUMMARY

Physarum rigidum grew and sporulated in pure culture at pH 4·2 on a partially defined medium containing: mineral salts, glucose, yeast extract, haematin, casein hydrolysate. The inoculum equiv. 0·22 mg. protein/25 ml. grew to equiv. 21·5 mg. protein/25 ml. in about 12 days. *Physarum flavicomum* varieties 1 and 2 also grew and sporulated on this medium. The yeast extract and haematin were essential for growth; the casein hydrolysate was not. Omission of glucose resulted in a 50% decrease in growth yield. Ethanol, galactose, glycerol, lactose, mannitol, mannose, potato starch, raffinose or sorbitol could replace glucose, but the growth yield was decreased. Fructose, inulin, sucrose and the pentoses and carboxylic acids tested did not support growth.

INTRODUCTION

Although there are over 450 known species of myxomycetes, the plasmodia of less than 10% of them have been grown in the laboratory in crude culture. Of these, only a few species have been grown on media which did not contain living or killed micro-organisms as a food source. According to Alexopoulos (1963) these include only *Licea flexuosa*, *Fuligo cinerea* and *Physarum polycephalum*. Ross (1964) reported the growth of *Physarella oblonga* and *Physarum flavicomum* on a partially defined medium but observed a gradual decline in the growth rate of the latter (Ross & Sunshine, 1965). Our previous paper (Henney & Henney, 1968) gave a genetic and morphological characterization of *P. rigidum* and *P. flavicomum* varieties 1 and 2. The present paper gives details for growth of these organisms in pure culture on a partially defined medium. They have been serially transferred on solid and liquid medium for more than 1 year with no alteration in characteristics or growth rate. This is the first report of the laboratory cultivation of *P. rigidum* in pure or crude culture. The composition of the medium developed is qualitatively and quantitatively different from those previously reported for other myxomycetes but is similar to that reported for *P. polycephalum* (Daniel & Rusch, 1961). Some of the nutritional requirements for *P. flavicomum* reported by Ross (1964) and Ross & Sunshine (1965) were not substantiated.

METHODS

Cultures. Axenic clones (myxamoebae) of the organisms studied were established by the methods previously described (Henney & Henney, 1968). Axenic plasmodia were produced by mixing two compatible clones together on half-strength corn meal agar

with the addition of formalin-killed *Aerobacter aerogenes* (or *Escherichia coli*). The cultures were incubated for several weeks at 22° after which time small plasmodia began to appear. Small amounts of sterile, long-cooking, ground oats were sprinkled onto the plasmodia. Incubation was continued until the plasmodia had reached a large size. Small pieces of the plasmodia were cut out, placed in the same bacteriological test media used for testing purity of the myxamoebae and incubated at 25° and 37° for 1 month. When bacterial growth developed in any of the test media, the plasmodial cultures were discarded and the entire procedure repeated. The axenic plasmodia thus developed could be maintained in pure culture for long periods of time. However, after several months of cultivation on oats, they accumulated large amounts of 'slime' and died unless transferred for a few times on the killed bacteria medium. The organisms also utilized oats overlaid with agar.

Plasmodia were cut from these plates, using a surgical blade sterilized in a flame, and transferred to sterile partially defined test media in glass Petri dishes. The cultures were subcultured to fresh media every 7 days. All transfers were made in a culture room with an air-filtration system and positive pressure. The Petri dishes were incubated at 22° and kept in the dark except during periods of examination.

Plasmodium was adapted to the liquid medium by transferring a block of agar carrying plasmodium to a sterile 500 ml. Erlenmeyer flask containing 50 ml. liquid medium. The flask was incubated inclined so that the liquid did not touch the plasmodium while it crawled onto the glass. The liquid was then allowed to touch the plasmodium until the latter floated, and the flask was aerated on a New Brunswick Scientific Co. (New Brunswick, New Jersey, U.S.A.) Model G 25 Gyrotory shaker at 170 rev./min. and room temperature (22° to 25°). The glass window of the shaker was covered to exclude light, and the pure cultures were exposed to light only during periods of examination. The plasmodium was soon converted into numerous microplasmodia which enabled replicate samples to be removed for inocula and analyses. Size of microplasmodial inoculum was measured as equiv. mg. protein/ml.

Stationary liquid cultures did not degenerate. The microplasmodial inoculum would form branching filaments, anastomose and crawl along the flask bottom while some floated to the top and adhered to the glass at the air/liquid interface. The plasmodia also grew well in 3 l. batches in a New Brunswick Fermentor Model FS 305 at about 1 l. air/min. at 200 rev./min. and room temperature.

Glassware. All glassware was cleaned with detergent and rinsed with tap water and glass-distilled water. Pipettes were cleaned in sulphuric + chromic acid cleaning solution and rinsed as above.

Water. Laboratory-distilled water (about 25 p.p.m. NaCl) was redistilled in a Corning Glass Works (Corning, New York, U.S.A.) Distillation Unit Model AG-3; the final product had less than 1 p.p.m. NaCl. This glass-distilled water was used throughout the work.

Reagents and substrates. All reagents were of Analytical Reagent grade. The substrates glucose, inulin, lactose, D-mannitol, raffinose, sorbitol and D-xylose were Difco (Detroit, Michigan, U.S.A.) products. Baker (Phillipsburg, New Jersey, U.S.A.) potassium acetate and purified potato starch, Eastman (Rochester, New York, U.S.A.) L-malic acid, Matheson (Norwood, Ohio, U.S.A.) fumaric acid, glycerol, sodium pyruvate and sucrose, and Calbiochem (Los Angeles, California, U.S.A.) ascorbic acid were used. L-Arabinose C.P. and D-galactose C.P. were obtained from Pfanstiehl

Laboratories (Waukegan, Illinois, U.S.A.), and sodium citrate U.S.P. from Allied Chemical (New York, N.Y., U.S.A.). Malonic acid, D-mannose and sodium succinate were from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.), and β-D-fructose, inositol, DL-lactate, and D-ribose from Sigma (St Louis, Missouri, U.S.A.).

Media. The composition of the medium finally developed is given in Table 1. Experiments at different pH values were made by changing the proportions of citric acid to potassium phosphate while maintaining the same total molarity. Growth was also determined at various concentrations of the basal salt mixture.

Table 1. *Medium for growth of Physarum rigidum*

Complete medium	
Basal salts mixture solution (pH 4·0)*	100 ml.
Trace elements solution†	0·1 ml.
Glucose (Difco)	5·0 g.
Yeast extract (Difco)	5·0 g.
N-Z Case (Sheffield)	5·0 g.
Agar (Difco)	25·0 g.
Glass-distilled water to	1000 ml.

Autoclave (121°, 15 min.) and cool to about 50°; add 1 ml. sterile 0·25% (w/v) haematin (haemin (Eastman, Rochester, New York, U.S.A.) in 1% NaOH; autoclaved); pour completed medium into sterile Petri dishes; final pH is about 4·2. Omit agar for liquid cultures.

* Basal salts mixture solution (pH 4·0). Add successively to about 700 ml. glass-distilled water (final volume to 1000 ml.) with stirring (g.): citric acid, 29·78; K_2HPO_4, 33·10; NaCl, 2·50; NH_4NO_3 anhydrous, 10·00; $MgSO_4.7H_2O$, 1·00; $CaCl_2.2H_2O$, 0·50.

† Trace elements solution. Dissolve successively in 95 ml. glass-distilled water with stirring (g.): citric acid, 5·00; $ZnSO_4.7H_2O$, 5·00; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1·00; $CuSO_4.5H_2O$, 0·25; $MnSO_4.H_2O$, 0·05; H_3BO_3 anhydrous, 0·05; $Na_2MoO_4.2H_2O$, 0·05; $Co(Cl)_2.6H_2O$, 0·05.

Difco Tryptone, Proteose-peptone, or Casamino acids and Sheffield (Norwich, New York, U.S.A.) N-Z Amine type A could be substituted for the Sheffield N-Z Case with no effect on growth yield. However, the first two protein hydrolysates led to abundant slime production on agar media. N-Z Case is a tryptic digest of casein in powder form and N-Z Amine is a pancreatic casein hydrolysate.

For liquid cultures, 50 ml. of media were used in 500 ml. Erlenmeyer flasks or 25 ml. in 250 ml. flasks; increased quantities of media resulted in decreased growth yields. A few drops of microplasmodial suspension (measured as equiv. mg. protein/ml.) were used as inoculum.

Protein analyses. Growth of the plasmodia was measured as an increase in protein content (mg./ml. culture) determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin (Armour, Kankakee, Illinois, U.S.A.) as a standard. Samples of the culture medium (2 ml.) or the inoculum samples were centrifuged at about 6000 *g* for 5 min. The yellow pigment was removed by incubating the pellet with 2 ml. acetone + 2 ml. 10% (w/v) trichloracetic acid solution for a few hours at room temperature, or overnight at 4°. The suspension was centrifuged at 12,000 *g* for 5 min., and the pellet dissolved in 0·4 N-NaOH by boiling for about 5 min. Samples were then analysed for protein content.

RESULTS

In general, the plasmodia did not grow well in plastic Petri dishes. This was especially true of *Physarum rigidum*, but *P. flavicomum* variety 1 grew better than the others under these conditions. This inhibition was quite significant and seemed to be due to

the lower humidity in the plastic dishes (because of less tightly fitting lids) rather than to the presence of a metabolic inhibitor.

Plate 1 shows cultures of *Physarum rigidum*, *P. flavicomum* variety 1 and *P. flavicomum* variety 2 on complete medium. There were distinct stable differences in morphology and colour among the plasmodia when grown under identical environmental conditions. The plasmodium of *P. rigidum* was yellow with a greenish tinge, very thin and spread out with an almost smooth edge. *P. flavicomum* variety 1 was almost orange, much thicker and grew in long finger-like projections. *P. flavicomum* variety 2 was lemon yellow, grew in a thin sheet but without the smooth edge or the long finger-like projections of the other two. Pure cultures of these organisms fused with themselves but not with each other. This extends the reports on crude cultures of plasmodia of different species which did not fuse (Alexopoulos, 1963) and different geographical isolates of the same species which did not fuse (Gray, 1945) to pure cultures as well. Carlile & Dee (1967) have recently reported similar results on fusion with pure cultures of *P. polycephalum*.

The organisms often sporulated after 10 days of incubation on the solid medium. *Physarum flavicomum* variety 1, in particular, formed fruiting bodies in at least 80% of the cultures. These spores, and those of *P. rigidum*, germinated yielding viable myxamoebae which increased in numbers and fused to form plasmodia in the presence of formalin-killed bacteria. Therefore the complete life-cycles of both *P. rigidum* and *P. flavicomum* have been completed in pure culture. *P. flavicomum* variety 1 also readily sporulated on solid medium in plastic Petri dishes.

The optimum temperature range for plasmodial growth of both species was between 22° to 25°; plasmodia did not survive above 30° or below 10°.

The only essential nutritional requirements for *Physarum rigidum* were the basal salts mixture, a carbohydrate, the yeast extract, and haematin (Table 1). Omission of the trace elements solution, the protein hydrolysate (N-Z Case) and of ammonium nitrate from the basal salts mixture had no effect on the final growth yield. The initial growth rate, however, was slower when a protein hydrolysate was omitted (Fig. 1). According to Difco Laboratories, Inc., Bacto Yeast Extract contains 13 amino acids. The complete medium is presented here (Table 1) since it may have application for other myxomycetes with more stringent growth requirements. The essential requirements for *P. rigidum* were also essential for *P. flavicomum*.

The optimum concentration of yeast extract was 0·5% (w/v), haematin 2·5 μg./ml. (Fig. 1, 2) and carbohydrate 0·5% (w/v). The concentration of essential nutrients was critical; in many instances a twofold increase in the optimum concentration resulted in death of the plasmodia. These concentration optima were not determined for *Physarum flavicomum*.

Physarum rigidum grew well at pH 3·0 to 4·2; 4·2 was usually selected since at the higher value haematin was more soluble and the agar solidified better. The pH value of the medium always increased during growth, regardless of the carbon source.

Although the basal salt mixture at the standard concentration of 0·77% (w/v) or $\frac{1}{2}$ concentration were equally effective, the higher concentration was selected because of its greater buffering capacity. A twofold increase or a tenfold dilution of the standard salt mixture resulted in about a 50% decrease in the maximum growth yield. *Physarum flavicomum* had the same optima of pH value and salt mixture concentration.

A typical growth curve for *Physarum rigidum* is shown in Fig. 3. After the maximum

plasmodial protein content was attained, there was a rapid decrease in this fraction. *P. flavicomum* variety 1 plasmodia developed a maximum protein content about 2·5 times greater than *P. rigidum*.

Older cultures accumulated abundant amounts of viscous materials when grown in shake culture with certain carbohydrates, especially in the presence of protein hydrolysates. Galactose, glucose, mannose and most carbohydrates containing them were most active in slime production. The plasmodia were separated from the growth media by centrifugation. The slime, collected by winding it on a glass rod from ethanol-layered supernatant fluid, was soluble in boiling water and gave no colour with iodine; its composition and production are being investigated. Presumably the casein hydrolysate was carbohydrate-sparing and enhanced polysaccharide production.

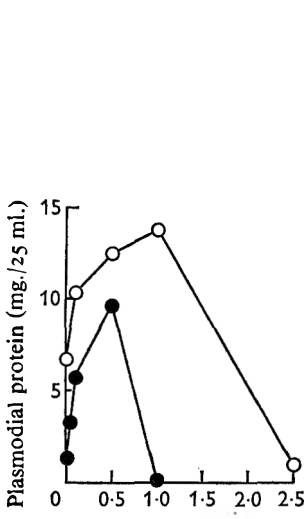


Fig. 1

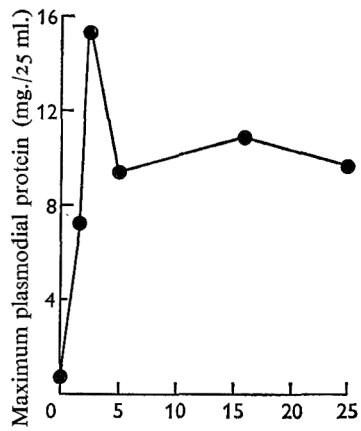


Fig. 2

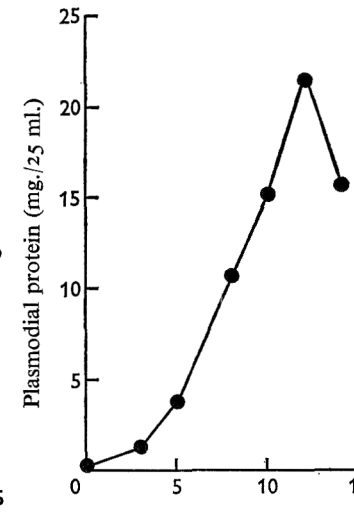


Fig. 3

Fig. 1. Yeast extract (●) and casein hydrolysate (N-Z Case) (○) requirements of *Physarum rigidum*. Shake cultures were analysed for protein after 8 days incubation in various concentrations of nutrients. The inocula contained 0·10 mg. protein/25 ml.

Fig. 2. Effect of variable concentrations of haematin on growth of *Physarum rigidum*. The shake cultures were analysed for protein at intervals for a total of 14 days. The maximum protein/25 ml. of medium attained during this time period is plotted. The inocula contained 0·13 mg. protein/25 ml.

Fig. 3. Growth of *Physarum rigidum* in shake culture on the complete medium. Two-ml. samples were removed and analysed for protein. The inoculum contained 0·22 mg. protein/25 ml.

Some of the carbon sources tested with *Physarum rigidum* are given in Table 2. All aldohexoses and alcohols tested, lactose, potato starch and raffinose gave good growth. Those substances which did not give good growth (at 0·5 %, w/v) included: arabinose, fructose and its polymer inulin, inositol, ribose, sucrose, and xylose. Acetate, ascorbate, citrate, fumarate, DL-lactate, L-malate, malonate, pyruvate and succinate were all toxic at a concentration of 0·5 % (w/v).

Table 2. *The effect of carbon sources on growth of Physarum rigidum*

Compound	Growth yield* (max. mg. protein/ 25 ml.)	Ratio of growth to growth with glucose	Compound	Growth yield* (max. mg. protein/ 25 ml.)	Ratio of growth to growth with glucose
Glucose	22·5 ± 1·1	1·00	Sorbitol	17·2 ± 1·3	0·76
Lactose	20·6 ± 2·4	0·92	Starch (potato)	16·8 ± 1·0	0·75
D-Mannitol	20·1 ± 0·1	0·89	Glycerol	15·0 ± 0·0	0·67
Ethanol	19·9 ± 0·4	0·89	D-Fructose	12·6 ± 1·4	0·56
D-Mannose	19·8 ± 1·7	0·88	Sucrose	12·0 ± 0·8	0·53
Raffinose	19·0 ± 1·1	0·84	D-Ribose	12·0 ± 0·1	0·53
D-Galactose	18·6 ± 1·5	0·83	None	11·8 ± 1·3	0·52

* Mean value ± average deviation from the mean based on four experiments.

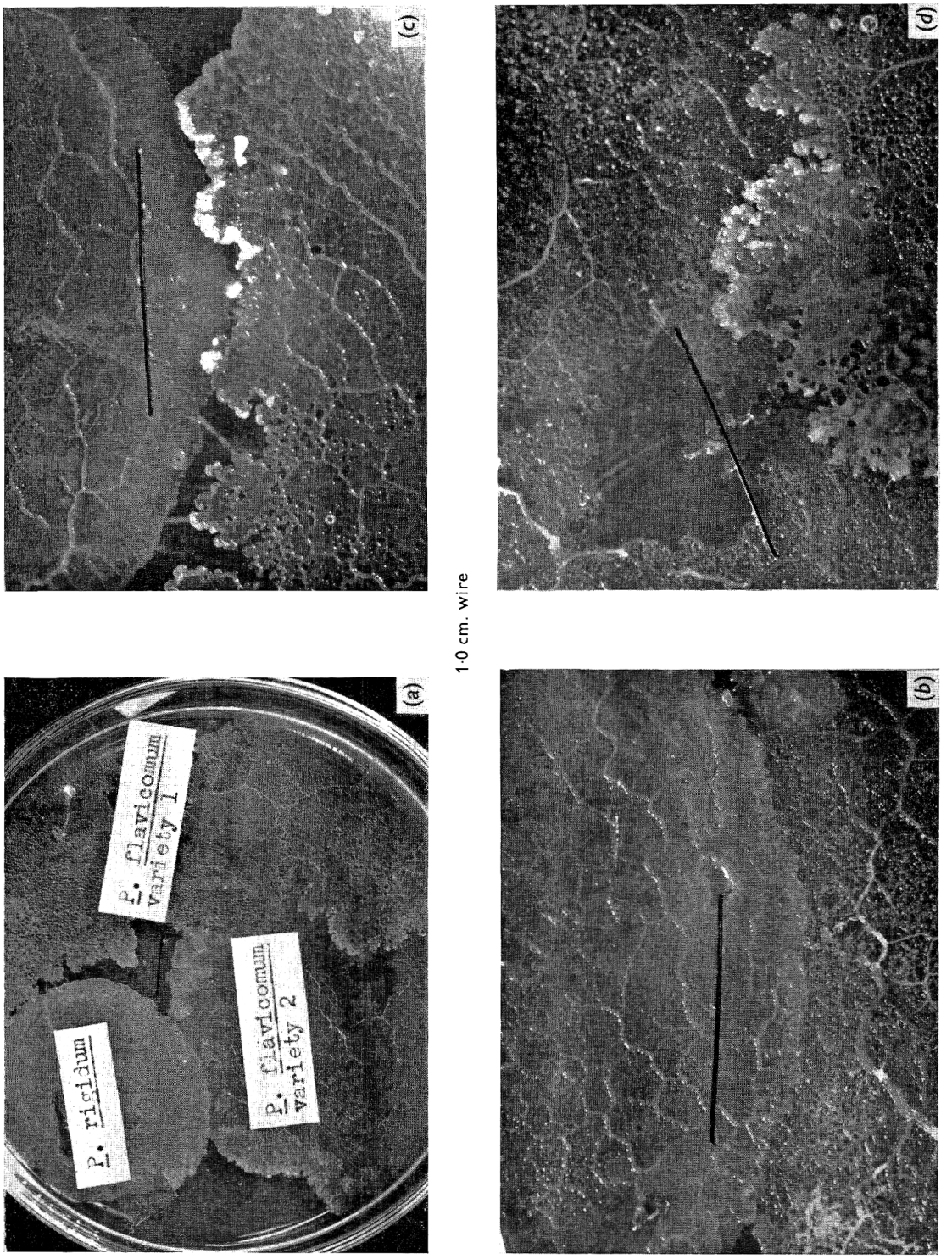
DISCUSSION

The growth requirements of *Physarum rigidum* differ from those of *P. polycephalum* as reported by Daniel & Rusch (1961). We found no absolute requirement for Tryptone and the optimum concentration of yeast extract was 3·3 times greater than for *P. polycephalum*. However, the optimum haematin concentration of 2·5 μg./ml. was the same for both organisms on a partially defined medium (Daniel, Kelley & Rusch, 1962). The growth rate and pH optimum were lower in the case of *P. rigidum* and important differences existed in the utilization of carbon sources. Daniel & Baldwin (1964) reported that *P. polycephalum* utilized fructose well, ethanol slowly, but that potato starch, D-galactose and glycerol supported little or no growth. Our results with these substrates and *P. rigidum* were just the reverse. Fructose and its polymer inulin were not utilized and the other compounds were utilized rather well.

The observations of an increase in pH value and formation of viscous polymers during growth of *Physarum rigidum* suggests that the organism might tend to polymerize available carbohydrates rather than actively catabolize them. The ready use of the sugar alcohols could provide an available source of biochemical energy in the form of reduced pyridine nucleotides.

The medium reported here for *Physarum flavicomum* is much simpler than, and quite different from, that indicated by Ross (1964) and Ross & Sunshine (1965). Most importantly, we found no requirement for corn meal agar, or for quinic or gallic acids either for growth or sporulation of this organism as reported by those authors. There were also significant differences in optima of pH value and salt concentration. It should be noted that we used the same variety of *P. flavicomum* as used by them (variety 1), but a different isolate. The present work was done with the PHILIPPINE II isolate while they worked with the AFRICA isolate. These two isolates formed a compatible allelic series (Henney & Henney, 1968).

We are grateful to Mr Jungkind for contributing his photographic talents. This research was supported by University of Houston grant no. F.R.S.P. 67–4.



H. R. HENNEY AND M. R. HENNEY

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EXPLANATION OF PLATE

Pure cultures on complete medium. (*a*) *Physarum rigidum*, *P. flavicomum* variety 1, and *P. flavicomum* variety 2; (*b*) *P. rigidum* and *P. flavicomum* variety 2; (*c*) *P. rigidum* and *P. flavicomum* variety 1; (*d*) *P. flavicomum* variety 2 and *P. flavicomum* variety 1. The length of the wire is 1·0 cm.

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Lipid Interrelationships in the Growth of *Paramecium aurelia*, Stock 299

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SUMMARY

In axenic medium with TEM-4T (a tartaric acid ester of beef tallow monoglyceride) as a source of fatty acids, regulation of the growth of *Paramecium aurelia*, stock 299, was achieved by varying the relative amounts of TEM-4T and stigmasterol. Optimal populations were reached in 7 days at 27° when the relative proportion of these lipids was 10:1 (w/w) TEM-4T:stigmasterol. Division was inhibited at ratios of 2:1 (w/w) or less. The inhibition was annulled by restoration of the 'optimal' ratios of TEM-4T:stigmasterol to the growth medium even after the protozoa had been exposed to 'inhibitory' ratios for periods up to 6 days. Growth was also inhibited when the relative proportions of TEM-4T:stigmasterol were adjusted to 2:1 (w/w) by the addition of TEM-4T to the culture after a 3-day period during which the organisms had been incubated with stigmasterol alone. No inhibition was observed when the TEM-4T:stigmasterol ratio was adjusted to 2:1 (w/w) by adding stigmasterol to the culture after a period of incubation with TEM-4T alone. It is suggested that under conditions of lipid imbalance in the medium stigmasterol interferes with the utilization of TEM-4T for growth.

INTRODUCTION

Paramecium aurelia requires both a sterol and a fatty acid for growth. The sterol requirement may be satisfied by stigmasterol and other structurally related sterols (Conner, van Wagtendonk & Miller, 1953; Conner & van Wagtendonk, 1955); the need for fatty acids may be met by oleic acid or any of a number of oleic acid-containing lipids, i.e. phospholipids, glycerides, Tweens and TEM's (tartaric acid esters of beef tallow monoglycerides; Miller & Johnson, 1960; Soldo, Godoy & van Wagtendonk, 1966*a*). As water dispersible sources of fatty acids, the TEM's are particularly effective in promoting growth and have gained increased application in the formulation of culture media for fastidious lipid-requiring micro-organisms (Shorb & Lund, 1959; Vogel & Hutner, 1961; Lee *et al.* 1962). When one of these substances, TEM-4T, was used as a source of fatty acids for the cultivation of *P. aurelia*, it was observed that regulation of growth could be achieved by altering the relative concentrations of TEM-4T and stigmasterol in the medium. Optimal growth, as measured by population density, was obtained at certain definite non-stoichiometric ratios of TEM-4T:stigmasterol, and division was arrested when these compounds were present in certain other proportions. The unusual nature of this response led us to a more detailed examination of this phenomenon. A preliminary account of this work has been published (Soldo, Godoy & van Wagtendonk, 1966*b*).

METHODS

Paramecium aurelia stock 299 (particle-free) was used in this work. The composition of the growth medium is given in Table 1. Details of the culture procedures have been described previously (Soldo *et al.* 1966*a*). TEM-4T was obtained from the Hachmeister Corporation, Pittsburgh, Pa.; stigmasterol was from Calbiochemicals, Inc., Calif. U.S.A., and was recrystallized once from 95% (v/v) ethanol in water. The lipids were dissolved in absolute ethanol and stored at −20° as concentrated (× 100) stock solutions until needed. The stock solutions were diluted with ethanol to the desired concentration and pipetted directly into the assay tubes. Ethanol was removed in vacuum at ambient temperatures. After the addition of full-strength culture medium the tubes were agitated vigorously by hand to aid in the dispersal of the lipids, capped with stainless-steel closures and sterilized at 121° for 20 min. In some experiments lipids from ethanolic stock solutions were diluted to the desired concentration with water, sterilized by autoclaving and added aseptically to the medium. The ethanol concentration in the final medium never exceeded 1% (v/v). Assays were started by adding 2 drops of a 7-day culture to each tube. The tubes were incubated in the dark at 27° for periods of a week or more. In certain experiments subcultures of each assay were made weekly.

Table 1. *Components of the medium for the cultivation of Paramecium aurelia stock 299*

TEM-4T and stigmasterol were dissolved in absolute ethanol as a × 100 concentrated solution and added to the medium before autoclaving. Vitamins were prepared as × 100 concentrated aqueous solutions. Both solutions were stored at −20° until ready for use. After the addition of all components, the medium was adjusted to pH 7·0 with 0·1 N-NaOH and sterilized at 121° for 20 min.

Component	Concentration	
	mg./ml.	μg./ml.
Proteose peptone	10	—
Trypticase	5	—
$MgSO_4.7H_2O$	—	500
TEM-4T*	—	100
Stigmasterol	—	5
Vitamins		
Calcium pantothenate	—	10
Nicotinamide	—	5
Pyridoxal HCl	—	5
Riboflavin	—	5
Folic acid	—	15
Thiamine HCl	—	15
Biotin	—	0·001
DL-thioctic acid	—	0·1

* Diacetyl tartaric acid esters of beef tallow monoglycerides.

RESULTS

In the absence of stigmasterol or TEM-4T, growth was decreased to near zero in the first weekly subculture and failed completely in the second (Table 2). Autoclaving the various components of the medium together or separately did not affect final population densities.

Growth was dependent upon the relative concentrations of stigmasterol and TEM-4T

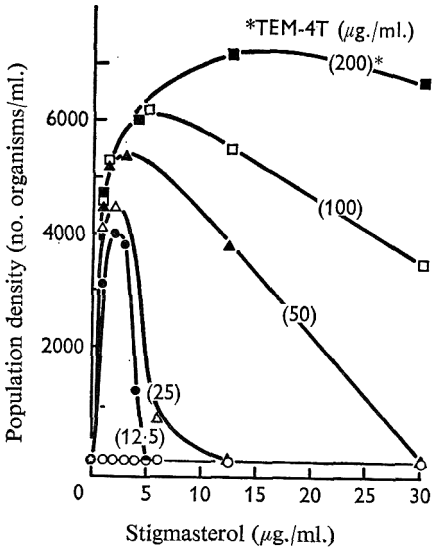

Fig. 1

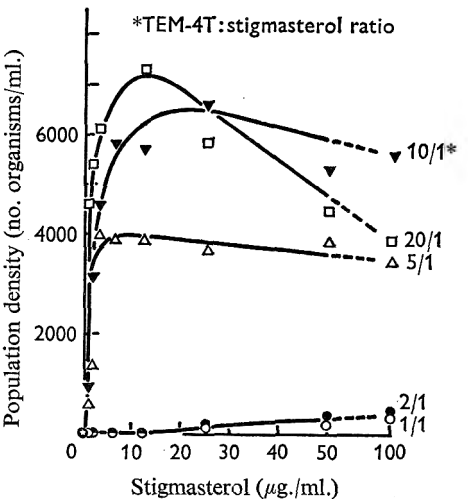

Fig. 2

Fig. 1. Growth response of *Paramecium aurelia* stock 299 to TEM-4T and stigmasterol.

Fig. 2. Growth response of *Paramecium aurelia* to various fixed ratios of TEM-4T: stigmasterol.

Table 2. *Growth response of Paramecium aurelia stock 299 to TEM-4T and stigmasterol*

	Number of weekly subcultures		
	1	2	3
	Population density†		
Component of medium			
Basal*	0	—	—
Basal + stigmasterol	1	0	—
Basal + TEM-4T	7	0	—
Basal + stigmasterol + TEM-4T (autoclaved separately)	102	98	99
Basal + stigmasterol + TEM-4T (autoclaved together)	100	100	100

* The basal medium consists of the components given in Table 1 minus TEM-4T and stigmasterol.

† Population density after 7 days expressed as % of control.

in the culture medium (Fig. 1). Small quantities of TEM-4T supported growth over a narrow range of stigmasterol concentrations. Increasing the concentration of TEM-4T in the culture medium permitted growth of the organisms over wider ranges of stigmasterol concentrations. At a given amount of TEM-4T in the medium the growth response was optimal at only certain concentrations of stigmasterol; at others growth was either suboptimal or failed completely. Figure 2 illustrates the growth response of *P. aurelia* to fixed ratios of TEM-4T:stigmasterol. Optimal growth was observed when the proportion of TEM-4T:sterol was 10:1. Ratios of TEM-4T:stigmasterol of 2:1 or less were inhibitory, although in some experiments small populations were noted at concentrations of stigmasterol of 25, 50 and 100 μg./ml.

The growth behaviour of the organisms cultivated in media containing proportions of TEM-4T:stigmasterol ranging from 10:1 (optimum) to 2:1 (inhibitory) was examined (Fig. 3). The ratios were obtained by maintaining the concentration of TEM-4T in the medium at a constant value (40 μg./ml.) and altering the amounts of stigmasterol. The rate of growth and the extent to which the organisms multiplied decreased as the ratio of these substances was decreased. At a ratio of TEM-4T:stigmasterol of 2:1, an actual decrease in population was observed. The length of the lag period of growth increased as the ratio of TEM-4T:stigmasterol decreased.

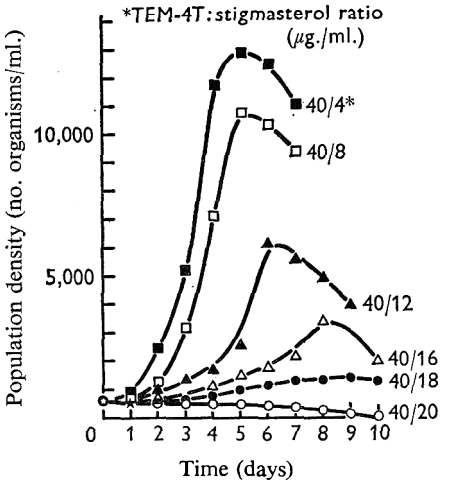

Fig. 3

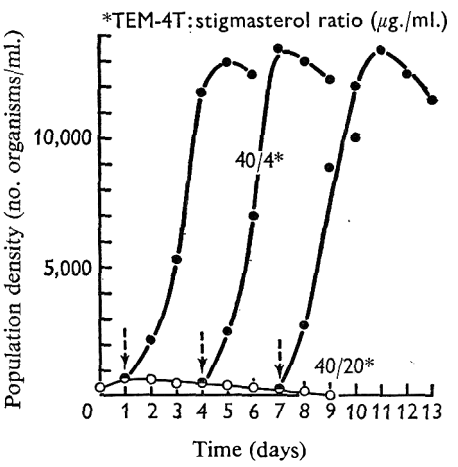

Fig. 4

Fig. 3. Growth curves of *Paramecium aurelia* in presence of various ratios of TEM-4T: stigmasterol.

Fig. 4. Annulment of growth inhibition of *Paramecium aurelia* after incubation with an 'inhibitory' ratio (2:1) of TEM-4T:stigmasterol. Arrows indicate points at which organisms were transferred to fresh medium containing an 'optimal' (10:1) ratio of TEM-4T: stigmasterol.

Inhibition of division brought about by placing the organisms in a medium containing a ratio of TEM-4T:stigmasterol of 2:1 was annulled, even after the organisms had remained under these inhibitory conditions for as long as 6 days, by transferring them to a fresh medium containing TEM-4T and stigmasterol in a ratio of 10:1 (Fig. 4). In these instances no lag period of growth was observed.

Neither TEM-4T nor stigmasterol added singly to the basal medium inhibited division (Fig. 5). In fact, the population increased significantly in the presence of either TEM-4T or stigmasterol even at concentrations two or three times higher than those indicated in Fig. 5. In all cases the initial increase in population was followed by a rapid decrease. 'Depleting' the organisms of most of their lipid reserves by maintaining them in a salt solution for 2 or 3 days before use as an inoculum had the effect of diminishing the rate at which the organisms divided and the extent to which they increased in number. These organisms when transferred to media containing a ratio of TEM-4T:stigmasterol of 40:20 (μg./ml.) did not divide. The population subsequently decreased steadily. Under these conditions the response of lipid-depleted and lipid-undepleted organisms was the same.

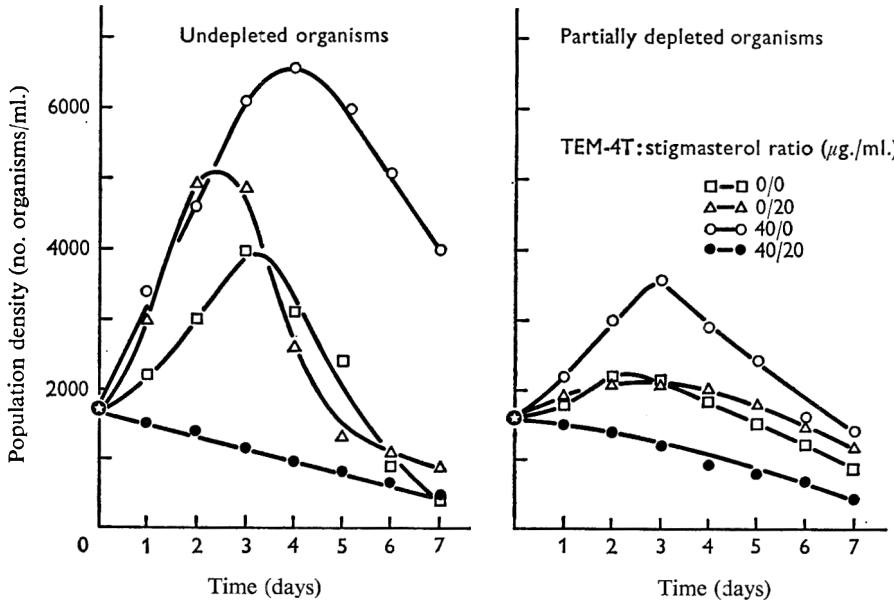

Fig. 5. Growth curves of *Paramecium aurelia* in presence of TEM-4T or stigmasterol alone. Depleted organisms were maintained in salt solution for 3 days before use as inoculum. The composition of the salt solution was as follows (mg./l.): NaCl, 30; $CaCl_2.2H_2O$, 300; $MgCl_2.6H_2O$, 200; KH_2PO_4, 30; $K_2HPO_4.3H_2O$, 45; adjusted to pH 7·0 with NaOH.

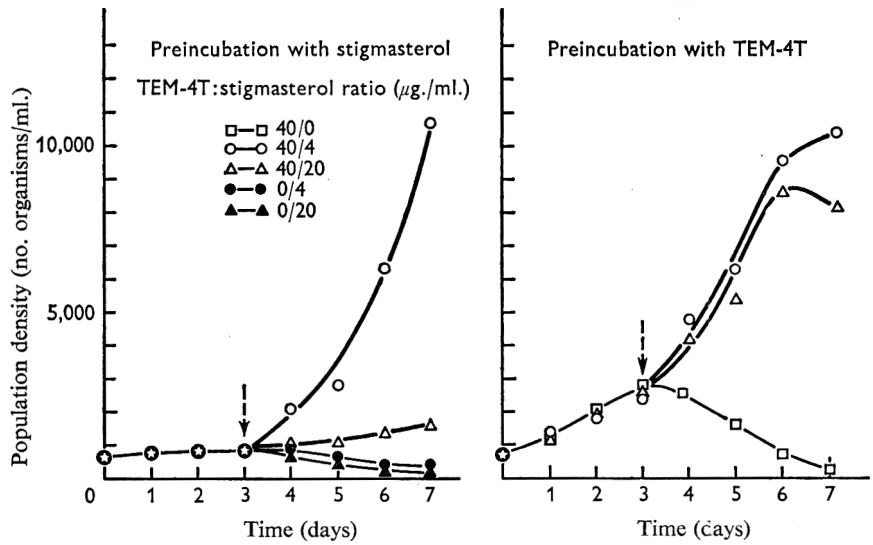

Fig. 6. Growth of *Paramecium aurelia*. Organisms (depleted of lipid by maintaining them in salt solution for 3 days) were incubated in the presence of stigmasterol (4 or 20 μg./ml.) or TEM-4T (40 μg./ml.) as indicated. After 3 days the ratio of TEM-4T: stigmasterol was adjusted (arrows) to either 40:4 μg./ml. (optimum) or 40:20 μg./ml. (inhibitory) by addition of appropriate amounts of each lipid directly to the flasks.

Figure 6 illustrates the effect of exposing the organisms to optimal and inhibitory ratios of TEM-4T:stigmasterol under conditions in which they have undergone a period of incubation with one of the lipids before the addition of the other. Organisms incubated with stigmasterol at 4 or 20 μg./ml. decreased steadily in population over a period of several days. Addition of an amount of TEM-4T on the third day to bring the ratio of TEM-4T:stigmasterol to 40:4 (μg./ml.) (optimum) resulted in an almost immediate and rapid increase in numbers, and maximum population density was reached in a few days. As expected, adjusting the relative proportions of TEM-4T:stigmasterol to 40:20 (μg./ml.) (inhibitory) by the addition of an appropriate quantity of TEM-4T to the culture prevented a significant increase in division. On the other hand, organisms incubated with TEM-4T only underwent one or two divisions, after which death occurred. Under these circumstances, the addition (on the third day) of an amount of stigmasterol calculated to bring about an inhibitory ratio (40:20) of TEM-4T:stigmasterol did not inhibit growth. Instead, the organisms divided at a rate and to an extent comparable to that of the control (TEM-4T:stigmasterol ratio = 40:4 μg./ml.).

DISCUSSION

In axenic medium TEM-4T and stigmasterol, nutrilites essential for the growth of *Paramecium aurelia*, inhibited growth when present in the culture medium in proportions of 2:1 (TEM-4T:sterol) or less. It seems unlikely that this inhibition can be accounted for on the basis of a toxicity of these two lipids. Relatively large amounts of TEM-4T or stigmasterol added singly to the culture medium did not inhibit growth. In fact, organisms not previously depleted of lipid responded to the addition of these two substances with a marked initial increase in population. It also seems unlikely that growth inhibition was due to toxic effects of material formed as a result of heat sterilization of TEM-4T+stigmasterol in the medium. When these compounds were sterilized separately and added aseptically to the medium in the same relative proportions, inhibition of growth did occur.

These results are not surprising since TEM's are considered to be relatively non-toxic sources of fatty acids, possibly because the fatty acids are in bound form and are released slowly into the medium (Shorb & Lund, 1959). Some sterols, on the other hand, have been reported to inhibit the growth of some micro-organisms which do not require sterols (Conner, 1959), but in general sterols are not inhibitory for micro-organisms which have a nutritional requirement for these lipids (Buetow & Levedahl, 1964). Some sterols have been reported to lessen or annul the toxic effects of several growth antagonists (Conner & Nakatani, 1958), and it has been suggested that they may serve to detoxify media containing fatty acids (Lwoff, 1951).

Completely inhibited organisms (TEM-4T:sterol ratio = 2:1) resembled starved organisms in some respects. They behaved as if deprived of an essential nutrilite and did not divide; they became increasingly less motile, decreased steadily in size and after a few days died. Similar changes took place when *Paramecium aurelia* previously grown under optimal conditions were suddenly placed in a non-nutritive environment, i.e. isotonic salt solution.

Complete arrest of division in the presence of relative proportions of TEM-4T:stigmasterol of 2:1 or less took place under the following conditions: (1) when these lipids were added at the same time to the culture medium; (2) when an appropriate

quantity of TEM-4T was added to the medium after a period of incubation with stigmasterol alone (Fig. 6). No inhibition was observed when stigmasterol was added to the medium after a period of incubation with TEM-4T alone. These results suggest that under conditions of lipid imbalance in the medium stigmasterol may interfere with the utilization of TEM-4T.

Oleic acid, which replaces TEM-4T for growth of this strain of *Paramecium aurelia* in crude axenic medium produces the best growth response when present in the growth medium at certain definite ratios of oleic acid:stigmasterol (Soldo & van Wagtendonk, 1967). These same lipids in other proportions inhibit growth.

Other workers reported the need for proportioned amounts of lipids for optimum growth. Shorb & Lund (1959) demonstrated that inhibition of growth of Trichomonas caused by linoleic acid (an essential nutrilite for this organism) could be overcome by the addition of adequate amounts of cholesterol to the culture medium. A definite balance of fatty acids and stigmasterol was necessary for the optimal growth of *Paramecium multimicronucleatum* (Miller & Johnson, 1960). Kodicek & Worden (1945) observed that inhibition of growth of *Lactobacillus helveticus* by unsaturated fatty acids could be reversed by the addition of cholesterol and related sterols. The ciliate *Tetrahymena corlissi* TH-X grew better in the presence of certain combinations of oleic acid and cholesterol than in the presence of either lipid alone (Holz, Wagner & Erwin, 1961).

As more complex protists are examined for their nutritional needs, it is to be expected that more exacting requirements for lipids will be uncovered. It may be important to test lipids at a variety of concentrations and relative proportions before deciding on their dispensibility or indispensibility. We have seen evidence here that lipid imbalances in the culture medium can result in the inhibition of growth.

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Taxonomy of the Aerobic Pseudomonads: *Pseudomonas diminuta* and *P. vesiculare*

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SUMMARY

Thirteen strains previously assigned to the species *Pseudomonas diminuta* and *P. vesiculare* were subjected to detailed characterization. Ten of these strains could be identified as *P. diminuta* and two as *P. vesiculare*. These two species can be readily distinguished by differences in their nutritional spectra, their growth factor requirements and their pigmentation. The DNA of the two strains of *P. vesiculare* contained 65·8 moles % guanine + cytosine (GC), and the DNA of the ten strains of *P. diminuta* from 66·3 to 67·3 moles %. One of the 13 strains examined had DNA of significantly lower GC content (62·2 moles %) and also differed in several phenotypic respects from both species; it is probably a monotypic representative of a third species, as yet unnamed.

All the strains examined share a series of distinctive properties, which justify their recognition as a special subgroup of aerobic pseudomonads. The defining characters of this subgroup include: monotrichous flagellation, with flagella of very short wavelength; a requirement for pantothenate, biotin and cyanocobalamin; a limited range of carbon sources; production of acid from primary alcohols by all strains that can utilize alcohols; inability to denitrify or to use nitrate as a nitrogen source; and accumulation of poly-β-hydroxybutyrate as an intracellular reserve.

INTRODUCTION

Pseudomonas diminuta was described by Leifson & Hugh (1954), on the basis of the study of three closely similar strains isolated from streams. The outstanding character of the species, according to these authors, was its unusual flagellar structure. The organisms were monotrichous and the flagellum very tightly coiled. The mean wavelength was only 0·6 μ, whereas the flagellar wavelength of most polar monotrichous bacteria is approximately 2 μ. Little other information concerning the properties of this species is available. Leifson & Hugh (1954) reported that it failed to produce acid from carbohydrates and gave negative responses to other routine biochemical tests, but formed acid from ethanol at concentrations as high as 5 % (v/v).

In the course of our taxonomic analysis of the aerobic pseudomonads (Stanier, Palleroni & Doudoroff, 1966; Redfearn, Palleroni & Stanier, 1966) ten strains that had been tentatively identified as members of this species on the basis of their flagellar structure were received from Dr H. Lautrop of the Statens Seruminstitut in Copen-

hagen. None of them could grow in simple defined media containing any one of a variety of organic compounds as sole carbon source; a preliminary study of their nutrition by Dr N. J. Palleroni suggested that the growth factor requirements were relatively complex.

We have now ascertained the minimal nutritional requirements of these bacteria, and have completed their phenotypic characterization by the methods previously applied to other aerobic pseudomonads (Stanier *et al.* 1966). In addition to the collection of strains received from Dr Lautrop, we have examined the type strain (ATCC 11568) and one other strain (ATCC 13184) of *Pseudomonas diminuta*, together with the type strain (ATCC 11426) of *P. vesiculare*. The latter organism was originally described as a *Corynebacterium* (Busing, Doll & Freytag, 1953), but was shown by Galarneault & Leifson (1964) to be a *Pseudomonas* sp., and Dr R. Hugh suggested to us (personal communication) that it might resemble *P. diminuta*. This inference has been confirmed: *P. diminuta* and *P. vesiculare* are closely similar species. They share a series of properties unusual among the aerobic pseudomonads so far examined.

METHODS

Origins of the strains examined

We shall refer to the strains by the numbers assigned to them in the collection of pseudomonads maintained in the Department of Bacteriology and Immunology of the University of California, Berkeley. Named strains of *Pseudomonas diminuta* (501, 502) and *P. vesiculare* (500) were obtained from the American Type Culture Collection; the others (ten in all) were provided by Dr H. Lautrop, Statens Serum-institut, Copenhagen, Denmark; their origins are indicated below. All these strains had been tentatively identified as *P. diminuta* by Dr Lautrop on morphological grounds.

500. *P. vesiculare* (Busing *et al.* 1953) Galarneault & Leifson (1964) (type strain). *Corynebacterium vesiculare* ATCC 11426. Isolated from the urinary-bladder epithelium of the leech *Hirudo medicinalis*.

501. *P. diminuta* Leifson & Hugh (1954; type strain). ATCC 11568. Isolated from a stream.

502. *P. diminuta* ATCC 13184. Isolated from buccal cavity.

230. Lautrop AB 102. Isolated from whooping-cough plate. 231. Lautrop AB 236. Isolated from spinal fluid. 232. Lautrop AB 265. Isolated from throat swab. 233. Lautrop AB 328. Culture received for identification. 234. Lautrop AB 359. Culture received for identification. 235. Lautrop AB 1122. Isolated from ear swab. 236. Lautrop AB 1224. Isolated from ascitic fluid. 237. Lautrop AB 1267. Isolated from urine. 238. Lautrop AB 1268. Isolated from pus from maxillary sinus. 239. Lautrop AB 1278. Isolated from blood culture.

Media

All media used in these studies were prepared with the standard mineral base described by Stanier *et al.* (1966). Yeast extract was prepared by adding Difco yeast extract 5 g./l. to the standard mineral base, and yeast agar by the further addition of Difco agar 20 g./l. These complex media were used for routine cultivation and maintenance of stocks.

Except in studies on specific growth factor requirements, chemically defined media were prepared by adding an appropriate organic carbon and energy source to the standard mineral base, together with a supplement of 15 vitamins (0·4 mg./l. each of: *p*-aminobenzoic acid, folic acid, biotin, nicotinic acid, nicotinamide, Ca pantothenate, riboflavin, thiamine, pyridoxal, pyridoxine, 2-methyl-1,4-naphthoquinone, choline, lipoic acid, haem, cyanocobalamin). Defined media for all strains except 230 and 500 were also supplemented with cystine 50 mg./l.

All cultures were incubated at 30° unless otherwise stated.

Nutritional requirements and gross physiology

The minimal growth factor requirements of all strains were determined by successive omissions of various amino acids or vitamins from a chemically defined medium containing the standard mineral base, 19 amino acids (L-cysteine, 50 mg./l. DL-threonine, 20 mg./l. and 10 mg./l. each of DL-lysine hydrochloride, DL-arginine, L-methionine, L-leucine, L-isoleucine, DL-valine, DL-phenylalanine, L-tyrosine, DL-tryptophan, L-histidine monohydrochloride, DL-glutamate-H_2O, L-proline, DL-aspartate, DL-alanine, glycine, DL-serine, and hydroxy-L-proline) and the 15 vitamins listed above. Sodium acetate (0·1 %, w/v, of the anion) was used as the principal carbon and energy source. After the minimal requirements for each strain had been ascertained, the suitability of the minimal medium was confirmed by making six successive serial transfers, using 0·01 ml. of inoculum per 5 ml. of medium.

One hundred and forty-six different carbon compounds were tested as carbon + energy sources by the replica plating method described by Stainer *et al.* (1966). Cystine and 15 vitamins (listed above) were added to the plates, before pouring, to give a final concentration in the agar media of about cystine 50 mg./l. and 0·4 mg./l. of each of the 15 vitamins. In all cases where growth on plates was questionable, utilization of the carbon sources in question was tested in chemically defined liquid media.

The ability to utilize nitrate as sole nitrogen source was determined by using the liquid standard mineral base medium, in which the $(NH_4)_2SO_4$ was replaced by KNO_3 (3 g./l.), and acetate (1 g./l.) was supplied as the carbon + energy source. Four successive transfers of each culture were made in the same medium, using an inoculum of 0·02 ml. into 5 ml. at each transfer.

Nitrate reduction was tested in yeast extract medium with the $(NH_4)_2SO_4$ replaced by KNO_3 (3 g./l.) and in the standard defined medium containing acetate as carbon source and KNO_3 (g./l.) either in addition to or in place of the $(NH_4)_2SO_4$. Cultures in 10 ml. of these media were incubated without shaking and subsequently examined for the production of nitrite by Trommsdorff's test (Feigl, 1954) and of ammonia by Nessler's reaction (Hawk, Oser & Summerson, 1947).

Denitrification was tested by the methods of Stanier *et al.* (1966) in yeast extract medium and in the standard mineral medium, with acetate (1 g./l.) carbon + energy source.

Endogenous reserve materials

Presumptive evidence for the accumulation of poly-β-hydroxybutyrate (PHB) as an intracellular reserve material was obtained by microscopic examination, as described by Stanier *et al.* (1966). Presence of the PHB was confirmed by isolating the granules

from hypochlorite-treated bacteria and subjecting them to the action of the PHB depolymerase of *Pseudomonas lemoignei*, as described by Delafield *et al.* (1965).

Quantitative tests for the accumulation of PHB and of glucose polysaccharide were done by growing the organisms in the defined mineral medium containing 0·15 % of either glucose or DL-β-hydroxybutyrate as carbon source. The bacteria were harvested and resuspended in 0·04 M-phosphate buffer (pH 6·8). Samples of the suspension were incubated with shaking at 30° in the presence or absence of carbon and nitrogen sources. After 6 hr, the bacteria were harvested by centrifugation and the dry weights determined. The PHB content of the bacteria was estimated by the method of Slepecky & Law (1960). Glucose polysaccharide content was measured by the use of the glucose oxidase-peroxidase reagent ('Glucostat', Worthington Biochemical Corp., Freehold, New Jersey), after hydrolysis of the bacteria with 4 N-H_2SO_4 at 100° for 2 hr.

Extraction and characterization of the orange pigments of strains 230 and 500

To determine the nature of the orange pigment(s) of strains 230 and 500, the bacteria were grown in yeast-extract medium with constant shaking for 48 hr and then harvested by centrifugation. The pigment was extracted from the bacteria by three successive treatments with 10 ml. absolute methanol/g. wet bacteria. Each extraction was done for 5 min. at room temperature and the bacteria removed by centrifugation. The final pellet of bacteria was colourless.

After addition of methanolic KOH to final concentration 3 % KOH, the methanolic extract was saponified for 10 min. at 50°. One-tenth volume of water was then added, and the extract acidified with concentrated HCl. Upon acidification, the colour of the extract changed from orange-yellow to orange-red. After the further addition of an equal volume of water, the pigments were repeatedly extracted with light petroleum (boiling range 30° to 60°, purified by passage through a silica gel column and dehydrated with anhydrous Na_2SO_4). The orange-red light petroleum extract was washed several times with distilled water to remove all traces of methanol. Columns of neutral alumina, diatomaceous earth, sucrose and anhydrous Na_2SO_4 were used in an attempt to separate these pigments. The columns were eluted with light petroleum containing increasing amounts of acetone. The pigments were treated with the Carr–Price reagent (Carr & Price, 1926) to establish their carotenoid nature.

Determination of the base composition of DNA

The mole % GC in the DNA of all strains was determined from buoyant density measurements in CsCl gradients (Mandel, 1966).

Miscellaneous tests

Morphological examinations were made on material from 24 hr yeast-extract agar slopes. Flagella were stained by the method of Leifson (1951).

The ability of strains to grow in yeast-extract medium at 4° and 41°, to hydrolyse gelatin, Tween 80, starch, and exogenously supplied poly-β-hydroxybutyrate, and to produce the 'egg yolk reaction' was determined as described by Stanier *et al.* (1966). All strains were tested for the ability to produce acid oxidatively from ethanol, *n*-propanol and various sugars, using the basal medium of Hugh & Leifson (1953) containing 1 % of each substrate.

The oxidase test was done with bacteria from 24 hr cultures grown on yeast-extract agar slopes, by the method described by Stanier *et al.* (1966).

The reduced/oxidized difference spectra of frozen preparations were determined with the Cary (model 14) recording spectrophotometer, equipped with a sensitive slide wire (total extinction span: 0·2 units) and an accessory device for maintaining sample cuvettes at the temperature of liquid nitrogen. The bacteria were harvested from cultures grown for 24 hr. in yeast-extract medium, washed, and resuspended in 0·03 M-phosphate buffer (pH 6·8) to a concentration of 20% (wet weight/volume). To establish a 'base line' for the instrument with the sample cuvette, two samples of the suspension were placed in plastic cuvettes, gassed with O_2 for 10 min. and then frozen in liquid nitrogen. The extinctions were compared over the range 390 to 650 mμ. The same cuvettes were then emptied and refilled with fresh suspensions. The reference cuvette was gassed with O_2 while the sample was allowed to become reduced either by endogenous metabolism or by the addition of dithionate. Both cuvettes were frozen in liquid nitrogen and their spectra compared (Sands, Gleason & Hildebrand, 1967).

RESULTS

Morphology

All the strains were Gram-negative rods (about 0·5 × 1 to 4 μ) with predominantly polar monotrichous flagella of short wavelength (0·6 to 1·0 μ), as previously demonstrated by Leifson & Hugh (1954), Galarneault & Leifson (1964) and Dr H. Lautrop (personal communication).

Growth factor requirements

All 13 strains could grow in a chemically defined medium containing acetate (0·1 %, w/v, of the anion) as the principal carbon+energy source, supplemented with 19 amino acids and 15 vitamins. The minimum growth requirements (Table 1) were determined by successive omissions of amino acids and vitamins from this medium. Every strain required three vitamins: pantothenate, biotin, cyanocobalamin. These supplements were sufficient to support growth of the type strain of *Pseudomonas vesiculare* (500) and of one other strain (230). The remaining 11 strains also required cystine, replaceable (although less effectively) by methionine. Three other sulphur compounds tested (thiosulphate, thioglycollate. DL-homocysteine) were unable to replace cystine.

Table 1. *Organic growth factor requirements of Pseudomonas strains examined*

Strain	Panto-thenate	Biotin	Cyano-cobalamin	Cystine
230, 500	+	+	+	−
231–239, 501, 502	+	+	+	+*

* Can be replaced by methionine, but growth is considerably slower; not replaceable by thiosulphate, thioglycollate or DL-homocysteine.

Utilization of carbon and energy sources

The diminuta group can use relatively few carbon compounds as principal carbon + energy sources: only 8 of the 146 compounds examined could be used by all strains (Table 2). Universal substrates include four amino acids (L- and D-α-alanine, L-gluta-

mate, L-proline) and four organic acids (acetate, pyruvate, butyrate, DL-β-hydroxybutyrate). Nineteen other compounds could be used by some, but not all, strains (Table 3). With respect to the utilization of these additional compounds, the type strain of *Pseudomonas vesiculare* (500) and strain 230 can be clearly distinguished from the remaining strains by virtue of their ability to use certain carbohydrates (D-glucose, D-galactose, maltose, cellobiose) and their failure to use L-histidine or pantothenate as carbon sources.

In view of the report by Leifson & Hugh (1954) that acid was produced from ethanol by *Pseudomonas diminuta*, this property was re-examined. With the exception of

Table 2. *Utilization of carbon compounds as substrates by the diminuta group*

Class of compounds	Compounds utilized by all strains	Compounds not utilized by any strains
Hydrocarbons		*n*-dodecane, *n*-hexadecane
Fatty acids	Acetate, butyrate	Isobutyrate, valerate, isovalerate, caproate, heptanoate, caprylate, pelargonate, caprate
Dicarboxylic acids		Oxalate, malonate, maleate, glutarate, adipate, pimelate, suberate, azelate, sebacate, eicosanedioate
Hydroxyacids	DL-β-hydroxybutyrate	D-Malate, D-(−)-tartrate, L-(+)tartrate, *meso*-tartrate, DL-lactate, glycollate, DL-glycerate, poly-β-hydroxybutyrate
Miscellaneous organic acids	Pyruvate	Citrate, laevulinate, citraconate, itaconate, mesaconate
Alcohols, glycols, polyalcohols		Methanol, isopropanol, *n*-butanol, isobutanol, geraniol, ethyleneglycol, propyleneglycol, 2,3-butyleneglycol, erythritol, mannitol, sorbitol, *meso*-inositol, adonitol, glycerol
Carbohydrates and sugar acids		D-Ribose, D-xylose, D-arabinose, L-arabinose, D-fucose, L-rhamnose, D-mannose, D-fructose, sucrose, trehalose, lactose, starch, inulin, gluconate, 2-ketogluconate, saccharate, mucate, salicin
Non-nitrogenous aromatic and other cyclic compounds		D-Mandelate, L-mandelate, benzylformate, benzoate, *o*-hydroxybenzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, phthalate, iso-phthalate, terephthalate, phenylacetate, phenylethanediol, naphthalene, phenol, quinate, testosterone
Amines		Methylamine, ethanolamine, benzylamine, putrescine, spermine, histamine, tryptamine, butylamine, α-amylamine
Aliphatic amino acids	L-α-alanine, D-α-alanine, L-glutamate	Glycine, β-alanine, L-threonine, DL-norleucine, L-valine, L-lysine, DL-arginine, DL-ornithine, DL-citrulline, DL-α-aminobutyrate, γ-aminobutyrate, DL-α-aminovalerate, δ-aminovalerate
Amino acids and related compounds containing a ring structure	L-proline	L-Tyrosine, L-phenylalanine, L-tryptophan, D-tryptophan, L-kynurenine, kynurenate, anthranilate, *m*-aminobenzoate, *p*-aminobenzoate
Miscellaneous nitrogenous compounds		Betaine, sarcosine, creatine, hippurate, acetamide, nicotinate, trigonelline

strains 502 and 235, all strains examined used both ethanol and *n*-propanol as carbon sources; these 11 strains likewise produced acid in complex media supplemented with 1 % (w/v) of either alcohol. Growth in such media ceased when the pH decreased to 4·7 (about 0·05 M-acid). In defined media containing ethanol as sole carbon source, strains 230 and 500 grew and produced acid with initial ethanol concentrations up to 5 % (v/v); with low initial concentrations, the acid formed subsequently disappeared. The other nine ethanol-oxidizing strains did not grow well in defined media with ethanol concentrations greater than 0·2 %, and produced little if any acid under these conditions.

Table 3. *Principal carbon sources utilized by some but not all of the strains of the diminuta group of Pseudomonas*

	Pseudomonas diminuta					*P. vesiculare*		
	502	236 238 239	231 232 233 501	237	234	500	230	Strain 235
D-Glucose	−	−	−	−	−	+	+	−
D-Galactose	−	−	−	−	−	+	+	−
Maltose	−	−	−	−	−	+	+	−
Cellobiose	−	−	−	−	−	+	+	−
Propionate	−	−	−	+	−	−	−	+
Succinate	−	−	−	−	+	+	+	−
Fumarate	−	−	−	−	+	+	−	+
L-Malate	−	−	−	−	+	+	+	−
Hydroxymethylglutarate	−	−	+	−	−	−	−	−
α-Ketoglutarate	−	−	−	−	−	+	+	+
Aconitate	−	−	−	−	−	−	+	−
Ethanol	−	+	+	+	+	+	+	−
n-Propanol	−	+	+	+	+	+	+	−
L-Serine	−	−	−	−	+	−	−	−
L-Leucine	−	−	−	−	+	−	−	+
L-Isoleucine	−	−	−	−	+	−	−	+
L-Aspartate	+	+	+	+	+	+	+	−
L-Histidine	+	+	+	+	+	−	−	+
Pantothenate	+	+	+	+	+	−	−	−

All strains that oxidized primary alcohols also accumulated acetone in complex media containing 1 % (v/v) isopropanol, but did not grow with either isopropanol or acetone as sole carbon source. Strains 502 and 235 did not produce acetone. None of the strains produced dihydroxyacetone from glycerol or fructose from either sorbitol or mannitol in complex media supplemented with these polyols.

Only strains 230 and 500 produced acid in complex media containing carbohydrates: acid was formed by these strains from glucose, galactose, L-arabinose, D-xylose. The last two sugars did not serve as carbon sources for the organisms. The metabolism of glucose and galactose has been shown to proceed through pathways previously established in *Pseudomonas saccharophila* (Entner & Doudoroff, 1952; MacGee & Doudoroff, 1954; DeLey & Doudoroff, 1957), except that either NAD or NADP can serve as hydrogen acceptor for the initial dehydrogenation of galactose. It has been found that accumulation of 2-keto-3-deoxyaldonic acids accounts for at least part of the acid produced from these sugars, as well as from pentoses, in complex media (unpublished results).

None of the strains grew chemolithotrophically in a mineral medium supplemented with growth factors and provided with CO_2 as principal carbon source and either molecular hydrogen or thiosulphate as possible electron donor.

Temperature ranges and growth rates

Three strains (235, 237, 502) grew in a yeast-extract medium at 41°. The remaining strains, which grew slightly or not at all at this temperature, grew at 37°. No strains produced visible turbidity after 10 days at 4° in yeast extract medium. More detailed experiments with the type strains of *Pseudomonas diminuta* (501) and *P. vesiculare* (500) showed that growth in yeast extract medium was slightly faster at 30° than at 37°. Quantitative determinations of growth rates for the two type strains at 30° showed that their growth was relatively slow, even in complex media. The generation times (determined turbidimetrically) and maximal yields of bacteria (by dry weight measurements) with several selected substrates in defined media and in a yeast extract medium are shown in Table 4. It will be noted that no defined medium supported the growth of either type strain at a rate comparable to the rate obtained with yeast extract. This may be because the yeast extract contains growth factors, other than the essential ones, that accelerate growth. The best single carbon sources were sugars for *P. vesiculare* (500) and the two stereoisomers of alanine for *P. diminuta* (501).

Table 4. *Generation times and bacterial yields in yeast extract and in defined media with different substrates at 30°**

	P. vesiculare 500		*P. diminuta* 501	
Substrate†	Generation Time (hr)	Yield‡	Generation Time (hr)	Yield‡
Yeast extract (1 g./l.)	3·2	.	3·1	.
Yeast extract (5 g./l.)	2·3	.	1·3	.
Glucose	7·4	985	.	.
Galactose	16·5	990	.	.
Ethanol	26	900	22	975
Acetate	12	800	10	750
Butyrate	21	790	19	760
DL-β-hydroxybutyrate	13·5	780	15	800
Succinate	29	790	.	.
L-malate	22	745	.	.
L-α-alanine	14	890	8·3	840
D-α-alanine	15·5	870	6·0	850
Proline	11	875	17	865

* Generation times and bacterial yields remained constant through three successive transfers of each culture in the same medium.

† Except for yeast extract, which was provided at 1 and 5 g./l., the initial concentration of all carbon sources in defined media was 1 g./l. (in the case of salts of organic acids, the concentration of the anion was 1 g./l.).

‡ Micrograms dry weight of cells per mg. of substrate carbon. Yields were not determined in complex media.

Endogenous reserve materials

Under the conditions described by Stanier *et al.* (1966), granules of poly-β-hydroxybutyrate (PHB) were observed in all the strains tested. Identity of the PHB was established in *Pseudomonas diminuta* (501), *P. vesiculare* (500) and strains 232 and 235 as described in Methods. During exponential growth in β-hydroxybutyrate medium, the

bacteria of strains 232 and 501 (*P. diminuta*) had a relatively high PHB content (about 10%) and a very low content of glucose polysaccharide (about 2%) on a dry-weight basis. In contrast, exponentially growing bacteria of strain 500 (*P. vesiculare*) contained essentially no PHB, but considerable amounts of polysaccharide (about 10% of dry weight in β-hydroxybutyrate medium and nearly 20% in glucose medium).

The carbohydrate and PHB contents of bacterial suspensions of strains 500 and 232 incubated in the presence and absence of suitable carbon sources as described in Methods are shown in Table 5. It can be seen that the carbohydrate content of strain 232 was essentially the same in the presence and absence of β-hydroxybutyrate, while the amount of PHB was greater in the bacteria oxidizing β-hydroxybutyrate. In the case of *Pseudomonas vesiculare* (500), both PHB and carbohydrate contents of the bacteria were greater in the presence of β-hydroxybutyrate or glucose as exogenous substrate. The presence of a nitrogen source stimulated the breakdown of the endogenous polysaccharide.

Table 5. *The intracellular accumulation of reserve materials**

Organism and growth substrate	Conditions of incubation	Bacterial weight (μg./ml.)		Final content of reserve materials (μg./ml.)	
		Initial	Final	Glucose polysaccharide	PHB
Strain 232	No carbon or nitrogen source	455	452	12	41
1·5 mg. DL-β-hydroxybutyrate/ml.	With 1·5 mg. DL-β-hydroxybutyrate/ml.; no nitrogen source	455	468	13	56
Strain 500	No carbon or nitrogen source	Not determined	1050	100	0
1·5 mg. DL-β-hydroxybutyrate/ml.	With 1·5 mg. DL-β-hydroxybutyrate/ml.; no nitrogen source	Same as above†	1255	144	156
Strain 500	No carbon or nitrogen source	548	544	106	0
1·5 mg. glucose/ml.	No carbon source; 1 mg. $(NH_4)_2SO_4$/ml.	548	517	74	0
	With 1·5 mg. glucose/ml.; no nitrogen source	548	628	131	54

* See section under Methods.
† Equal samples of a bacterial suspension were used.

Other physiological properties

All the strains are obligate aerobes, and incapable of denitrification in either complex or chemically defined media. Only one strain (502) reduced nitrate to nitrite; it produced nitrite in all three of the media (defined and complex) tested.

None of the strains hydrolysed starch, gelatin, Tween 80, or exogenously supplied poly-β-hydroxybutyrate in 48 hr; none produced the 'egg yolk reaction'.

Oxidase reaction and reduced/oxidized difference spectra

With the exception of 230, 235 and 500, all strains gave a strong positive oxidase reaction with *N,N'*-dimethyl-*p*-phenylenediamine; these three exceptional strains gave a much weaker but still perceptible reaction. The reduced/oxidized difference spectra of four strains, two of which gave a strong oxidase reaction (232, 501) and two of which gave a weak reaction (235, 500), were determined and found to be qualitatively similar. All showed a peak at 628 mμ corresponding to a cytochrome a_2 component, another at 598 mμ corresponding to cytochrome a_1, and a peak at 557 mμ and shoulder at 562 mμ characteristic of a *b*-type cytochrome. All strains also showed the presence of a *c*-type cytochrome with a peak at 551 mμ, except for strain 235 in which the absorption maximum was displaced to 549 mμ.

Significant quantitative differences were observed, however, between strains that gave a strong oxidase reaction and those that gave a weak one. In the former, the amount of cytochrome *c* was much greater than in the latter, while the amounts of cytochromes *a* and *b* were approximately the same in both groups. The height of the *c* peak relative to that of the *b* peak was 0·92 and 1·0, respectively, in strains 232 and 501, but only 0·66 and 0·62, respectively, in strains 235 and 500. These results lend further support to the conclusion that the oxidase test is essentially a test for the presence or absence of a *c*-type cytochrome in the terminal respiratory chain (Baumann, Doudoroff & Stanier, 1968; Stanier *et al.* 1966; Sands *et al.* 1967).

Characterization of the pigments of strains 230 and 500

Attempts to separate the orange-red pigments extracted from strains 230 and 500 by chromatography on neutral alumina, diatomaceous earth or sucrose were unsuccessful. A degree of separation was, however, achieved by adsorption on a column of anhydrous Na_2SO_4 and elution with light petroleum containing increasing amounts of acetone; a yellow band was eluted from the column with 2·5% acetone and an orange band with 10% acetone. The absorption spectrum of the yellow pigment in pure light petroleum showed two major peaks, at 426 and 452 mμ, and shoulders at 403 and 477 mμ. The orange pigment had an absorption maximum at 488 mμ, a peak at 515 mμ with a shoulder at 553 mμ, and a minor peak at 397 mμ.

The pigments gave a blue colour with the Carr–Price reagent, and thus appear to be carotenoids.

Table 6. *DNA Composition (moles % GC) of the strains of the diminuta group of Pseudomonas*

Moles % GC*	Strains
62·2	235
65·8	230, 500
66·3	231, 502
66·9	233, 234, 237, 238
67·3	232, 236, 239, 501

* Raw mean of two determinations.

Base composition of DNA

The base compositions of the DNA's in all strains examined fell in the range previously shown (Mandel, 1966) to be characteristic of aerobic pseudomonads (Table 6). The value for strain 235 (62·2 moles % GC) is significantly lower than those for the remaining 12 strains, which are all in the narrow range 65·8 to 67·3.

DISCUSSION

The 13 strains examined possess a considerable number of morphological, nutritional and physiological characters in common. These shared characters appear to justify their recognition as a special subgroup within the genus *Pseudomonas*, as defined by Stanier *et al.* (1966). We shall term it the 'diminuta group'. Some of the characteristic properties of the diminuta group are: (1) possession of polar monotrichous flagella of short wavelength; (2) requirement for pantothenate, biotin and cyanocobalamin; (3) inability to use nitrate as a sole nitrogen source, to perform denitrification or, with one exception (strain 502), to reduce nitrate to nitrite: (4) positive oxidase reaction of variable intensity (5) possession of a_1, a_2; *b*, and *c* cytochrome components; (6) accumulation of poly-β-hydroxybutyrate as an endogenous reserve material; (7) a very limited nutritional spectrum, as compared with most other pseudomonads; and (8) inability to hydrolyse starch, gelatin, Tween 80, exogenously supplied poly-β-hydroxybutyrate, or to produce the 'egg yolk reaction'.

It should be noted that most of these strains had been provisionally grouped together on the basis of the first-mentioned character by Dr H. Lautrop, and that Dr R. Hugh had suggested a possible relationship between *Pseudomonas vesiculare* and *P. diminuta* on the same grounds (personal communication). However, the short wavelength of the flagella is not alone sufficient to characterize this group. A study of the hydrogenomonads (Davis, 1967) revealed the existence of other strains which have a flagellar structure like that of the diminuta group, but which do not share the distinctive physiological properties of this group.

On phenotypic grounds, eight of the ten strains tentatively identified as *Pseudomonas diminuta* by Dr H. Lautrop and strain 502, so identified by Dr Hugh, can be grouped with the type strain (501) of *P. diminuta*. One of Lautrop's strains (230) is almost identical with the type strain (500) of *P. vesiculare*. Lautrop's strain 235 is the only one that cannot be readily included in either species.

Several features distinguish *Pseudomonas diminuta* from *P. vesiculare* (Table 7). The major differences are the requirement for cystine by all strains of *P. diminuta*, and utilization of sugars and the production of carotenoid pigments by *P. vesiculare*.

Although the ten strains that we have assigned to *Pseudomonas diminuta* show minor phenotypic differences (Table 3), we do not consider that these differences justify a taxonomic subdivision. The most aberrant strains are 502 and 234. Strain 502 does not oxidize alcohols, and is the only strain in the entire collection that produces nitrite from nitrate. Strain 234 uses six carbon sources that are not used by the other nine strains. The inability of strain 502 to use alcohols may reflect the absence of only one enzyme possessed by the other strains. Three of the carbon sources utilized uniquely by strain 234 are the closely related compounds succinate, fumarate and L-malate; this nutritional feature probably reflects possession of a single permease.

Strain 235 resembles *Pseudomonas diminuta* in its growth-factor requirements, its ability to use L-histidine and its inability to use sugars or to produce carotenoid pigments. It resembles *P. vesiculare* in its weak oxidase reaction, ability to use α-ketoglutarate and inability to use pantothenate as sole carbon source. Strain 235 also differs from all other strains studied (with the exception of *P. diminuta* strain 502) in its inability to oxidize alcohols. The GC content of the DNA of strain 235 is markedly lower than that of the remaining strains. These differences are summarized in Table 7. It seems probable, accordingly, that strain 235 is a monotypic representative of a third species of the group; for the time being, we prefer to leave it unnamed.

Table 7. *Characters distinguishing Pseudomonas vesiculare, P. diminuta, and strain 235*

Characters	*P. vesiculare*	*P. diminuta*	Strain 235
GC content of DNA (moles %)	65·8	66·3–67·3	62·2
Cystine requirement	−	+	+
Oxidase reaction	Weak	Strong	Weak
Carotenoid production	+	−	−
Utilization of carbon sources			
D-glucose, D-galactose, maltose, cellobiose	+	−	−
α-Ketoglutarate	+	−	+
L-Histidine	−	+	+
Pantothenate	−	+	−
Growth with 5% ethanol in defined medium	+	−	−

The diminuta group shares relatively few properties with the fluorescent pseudomonads, which must necessarily comprise the central cluster of the genus *Pseudomonas*, since they include its type species, *P. aeruginosa* (Stanier *et al.* 1966). It is therefore necessary to discuss briefly the taxonomic reasoning which has led us to include the diminuta group in the genus *Pseudomonas*. In some respects, the diminuta group resembles the polarly flagellated under-oxidizing acetic acid bacteria, now placed in a separate genus, *Acetomonas* (Leifson, 1954) or *Gluconobacter* (Asai & Shoda, 1958). The features common to the two groups are the production of acids in complex media containing ethanol or aldose sugars, the production of acetone from isopropanol, and the multiple requirements for growth factors, which include pantothenate. However, under certain conditions the fluorescent pseudomonads also produce acids from ethanol (Stanier, 1947) and from glucose, and of the three groups in question only *Acetomonas* species are incapable of oxidizing acetate. In terms of over-all phenotype (flagellar morphology, nutritional spectra, relative acid tolerance, the chemical nature of the intracellular and extracellular pigments and of the intracellular reserve materials) the diminuta group appears to be at least as distinct from the fluorescent group as it is from the polarly flagellated acetic acid bacteria. One solution of this taxonomic problem would be to make extensive generic subdivisions among the aerobic pseudomonads, reserving the genus *Pseudomonas* for the fluorescent types. We are not opposed in principle to this solution, but, in our judgement, knowledge of the entire group is still insufficient to determine the bases on which the series of genera could best be created. As an interim taxonomic solution, we therefore favour the maintenance of a single

genus *Pseudomonas*, broadly defined (Stanier *et al.* 1966). Both the diminuta group and the polarly flagellated acetic acid bacteria now classified in the genus *Acetomonas* can be satisfactorily accommodated in it as generic subgroups.

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Biochemical and Genetic Studies with Lysine + Methionine Mutants of *Escherichia coli*: Lipoic Acid and α-Ketoglutarate Dehydrogenase-less Mutants

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SUMMARY

A selection procedure was developed for the isolation of mutants of *Escherichia coli* which require lipoic acid, acetate + succinate, or lysine + methionine for aerobic growth with glucose. The properties of the mutants requiring lipoic acid (*lip*$^-$) were compared with those of *suc*$^-$ mutants which lack α-ketoglutarate dehydrogenase and require succinate or lysine + methionine. Genetic analysis by conjugation with Hfr and F′ donors indicated that the genetic loci of some 36 independently isolated *lip*$^-$ mutants are confined to a small segment of the *E. coli* chromosome between the *purE* and *suc* sites. By using phage P1 no cotransduction of *lip* with *suc*, *gltA* or *purE* could be demonstrated, but *suc*, *gltA* and *gal* were cotransducible and the relative order of these sites was determined.

INTRODUCTION

The requirement for lysine + methionine exhibited by certain mutants of *Escherichia coli* for aerobic growth in glucose minimal medium stems from a metabolic block in the synthesis of succinylCoA which is necessary for the succinylation reactions found in the biosynthesis of lysine (Gilvarg, 1963) and methionine (Rowbury & Woods, 1964). It is now apparent that this phenotype can be the result of mutations which affect a variety of different genes. Davis *et al.* (1959) isolated a mutant which had an alternative requirement for either succinate or lysine + methionine + threonine and which was later shown to lack the decarboxylase component of the α-ketoglutarate dehydrogenase system (Hager & Kornberg, 1961) and not to require threonine (Kaplan & Flavin, 1964). A mutant isolated by Back & Westaway (1962) which required lysine + methionine was subsequently shown to respond better to acetate + succinate and to lipoic acid (Vise & Lascelles, 1967). The primary lesion was attributed to an inability to synthesize lipoic acid which in turn prevented the synthesis of acetylCoA and succinylCoA by the lipoic acid-dependent pyruvate and α-ketoglutarate dehydrogenase systems. The 4-hydroxybenzoic acid requirement of multiple aromatic mutants can also be replaced by either succinate or lysine + methionine and to a lesser degree by α-ketoglutarate and glutamate (Davis, 1955; Jones & Lascelles, 1967). Recently, two mutants of *Aerobacter aerogenes* requiring either lysine + methionine or succinate have been described (Stouthamer, 1967). One of the mutants (42) lacked α-ketoglutarate decarboxylase and the other (19), which differed in being able to use succinate as a sole carbon source, appeared to have an impaired capacity for oxidizing pyruvate and α-ketoglutarate. A feature common to all lysine + methionine mutants is the ability to

grow anaerobically on glucose without supplements and this can be explained by the presence of fumarate reductase and the phosphoroclastic reaction, which supply the organism with succinate and acetate, respectively, under anaerobic conditions, but are repressed by aerobiosis (Hirsch, Rasminsky, Davis & Lin, 1963; Henning, 1963).

Genetic maps of *Escherichia coli* K12 indicate the presence of *lys*+*met* (Taylor & Thoman, 1964) and *suc* (Jacob & Wollman, 1961) loci in the same region of the chromosome. The *lys*+*met* mutant of Taylor & Thoman (AT 2036) was thought to correspond to the mutant isolated by Back & Westaway; however, it has been found to respond to succinate but not lipoic acid (see below). Thus it appears likely that the *lys*−*met* and *suc* are similar and correspond to the same type of succinate or lysine+methionine mutant, presumably the type isolated by Davis *et al.* (1959). During studies initiated with the purpose of investigating the biosynthesis of lipoic acid in *E. coli*, about which virtually nothing is known, attempts were made to isolate nutritional mutants blocked at different stages in the biosynthetic sequence. At the same time, a variety of other mutants requiring lysine+methionine or succinate were isolated. The present paper describes work with two types of mutant, lipoic acid-requiring strains (*lip*$^-$) and α-ketoglutarate dehydrogenase-less strains (*suc*$^-$).

METHODS

Organisms. The characteristics and sources of some of the strains of *Escherichia coli* K12 used in these studies are shown in Table 1. XG3 is a *lys*$^+$, *met*$^+$ derivative of X478 (Berg & Curtiss, 1967); it was obtained by successive transductions with phage P1kc using W1485 as the donor. In addition, W1485 *gal*$^-$ was obtained from W1485 by treatment with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine. From this, W1485 *glt*$^-$ was prepared by cotransduction of *gal*$^+$ and *glt*$^-$ from strain K2-1-4 (a citrate synthase mutant obtained from Professor H. L. Kornberg) using phage P1vir1 (Ikeda & Tomizawa, 1965). Another glutamate-requiring strain, W620 (F$^-$, *glt*$^-$, *galK*$^-$, *ura*$^-$, *str*^{r}) was kindly provided by Dr E. L. Wollman (Reissig & Wollman, 1963). Enzymic analysis at pH 8·0 by the method of Dixon & Kornberg (1959) showed that cell-free extracts of W1485 *glt*$^-$ and W620 lack citrate synthase. These organisms and the *lip*$^-$ and *suc*$^-$ derivatives to be described were stored as stabs in Bacto-Nutrient broth+0·8% agar in corked tubes sealed with paraffin wax. Running stocks on L-agar slopes or plates were subcultured every 6 weeks (18 hr, 37°) and kept at 2°. Unless otherwise stated, all cultures were incubated aerobically.

Media. Two minimal salts media were used, medium E of Vogel & Bonner (1956) prepared at 50 times the final strength and a citrate-free basal medium containing (g./l. final strength): K_2HPO_4, 6; $NaH_2PO_4.2H_2O$, 2·5; NH_4Cl, 2; Na_2SO_4, 0·15 and $MgCl_2.6H_2O$, 0·1, prepared as two stock solutions, salts minus $MgCl_2$ at 50 times final strength and a solution of $MgCl_2$ at 1000 times the final strength. After sterilization the concentrated salts were added to the otherwise complete sterile bulk media. The concentrations of carbon sources were: glucose, 0·2%; galactose or lactose, 0·5%; sodium succinate, 50 mM; sodium acetate, 50 mM. All media were supplemented with thiamine-HCl (5 μg./ml.) and when required other additions were made to give final concentration (μg./ml.): L-arginine-HCl, 30; monosodium L-glutamate, 300; glycine, 40; L-histidine-HCl, 35; L-leucine, 30; L-lysine-HCl, 80; L-methionine, 20; L-proline.

25; L-threonine, 25; L-tryptophan, 20; adenine sulphate, 35; anthranilic acid, 15; DL-lipoic acid, 0·005; streptomycin sulphate, 200; thymidine, 4; uracil 35. When used as growth factors acetate and succinate were added to final concentration 4 mM. Media were solidified when necessary with Difco Bacto Agar (1·5 %) and for the selective media used in transduction studies solid media were also supplemented with Bacto Nutrient Broth (0·2 %, v/v). The complete medium used for growth and maintenance of the organisms was L-broth and L-agar (Lennox, 1955). The presence of fermentation markers was tested with two indicator media containing the appropriate sugar: the EMB agar of Lennox (1955) in which the eosin and methylene blue were replaced by bromothymol blue (18 μg./ml.) and minimal medium with the same concentration of bromothymol blue. All complex media were routinely supplemented with DL-lipoic acid (5 mμg./ml.) unless organisms depleted of the factor were specifically required.

Table 1. *Strains of Escherichia coli K12*

Strain	Mating type	Characteristics*		Source or reference
AB1325	F⁻	*proA⁻ lacY⁻ T_6^r gal⁻ purB⁻ his⁻ str^r mtl⁻ xyl⁻ thi⁻*		Taylor & Thoman (1964)
AT2036	F⁻	*proA⁻ lacY⁻ T_6^r lys+met⁻ gal⁻ his⁻ str^r xyl⁻ argA⁻ thi⁻*		Taylor & Thoman (1964)
XG3	F⁻	*leu⁻ ara⁻ lac⁻ proC⁻ T_6^r purE⁻ trp⁻ str^r xyl⁻ thi⁻*		Derivative of x478 (Berg & Curtiss, 1967)
W1485	F⁺	prototroph		C. Yanofsky
T3A58	F⁺	*trpE⁻, A⁻*		C. Yanofsky
H	Hfr	*str^s*;	*o, thr leu proA, B, C gal ...F*	W. Hayes
C	Hfr	(λ) *met⁻ str^s*;	*o, purE proC lac ...F*	W. Hayes
B11	Hfr	*met⁻ str^s*;	*o, proC purE gal ...F*	R. H. Pritchard
R4	Hfr	*metA⁻* or *B⁻ str^s*;	*o, proB, A leu thr ...F*	R. H. Pritchard
AT2572	Hfr	*str^s thi⁻*;	*o, his gal proC, B, A ...F*	R. H. Pritchard
ORF-1	Primary F′	prototroph	*o, purE proC lac ...F*	Berg & Curtiss (1967)
ORF-4	Primary F′	*ser/gly*;	*o, purE proC lac ...F*	Berg & Curtiss (1967)
ORF-8	Primary F′	*leu⁻ thy⁻*;	*o, purE proC lac ...F*	Berg & Curtiss (1967)
ORF-15	Primary F′	*leu⁻ thy⁻*;	*o, proC purE lac ...F*	Berg & Curtiss (1967)
W3101 (F_2-*gal*)	Secondary F′	*galK⁻/F-gal⁺*;	*o, gal ...F*	F. Jacob

* The genetic markers are arranged in the order they occur on the chromosome, the abbreviations adopted are those in common use; − indicates inability to synthesize or utilize; +, ability to synthesize or utilize; s, sensitive; r, resistant. The polarity of marker entry for the donor strains is also indicated, *o* representing the origin of transfer.

Isolation of mutant organisms. Mutations leading to a requirement for lipoic acid and succinate were induced in several strains of *E. coli* using *N*-methyl-*N′*-nitro-*N′*-nitrosoguanidine (MNNG) as the mutagen and a modification of the method described by Adelberg, Mandel & Chen (1965). Log phase L-broth cultures of the parent organism (5 ml.) were washed once and resuspended in the same volume of tris-maleate buffer (0·1 M, pH 6·0). Samples (0·2 ml.) of a freshly prepared solution of MNNG (2·5 mg./ml. of sterile water) were added and the suspensions incubated at 37° for 30 min. The organisms were then washed twice by centrifuging and resuspending in the same volume of cold minimal medium. Samples (0·5 ml.) were diluted into 5 ml. of glucose minimal medium containing the necessary supplements for the treated organism plus lipoic acid (1 mμg./ml.) and the cultures incubated at 37° for 18 hr with shaking. The expressed cultures were then washed twice and an aliquot inoculated into 10 volumes of glucose minimal medium without lipoic acid, and shaken at 37°. The purpose of this step was to deplete the organisms of lipoic acid. After 3 to 4 hr, when the population had increased 2- or 3-fold, the bacteria were resuspended in minimal

medium and samples (0·05 ml., approximately 2×10^7 organisms) were inoculated into 5 ml. of glucose minimal medium + benzylpenicillin (300 units/ml.) and incubated at 37° for 8 or 18 hr. After the penicillin selection, suitable dilutions of the treated culture were spread on glucose minimal medium supplemented with acetate + succinate to obtain 50 to 150 colonies per plate, and mutants were detected by printing on to glucose minimal media by the replica plating technique (Lederberg & Lederberg, 1952). Prospective mutant colonies were picked, diluted in saline and inoculated as small patches on glucose minimal + acetate + succinate plates (16 patches/plate). After incubation, these served as master plates for further replica plating tests for the ability of the isolates to grow aerobically with glucose alone, glucose supplemented with lipoic acid, acetate, succinate, acetate + succinate or lysine + methionine, with acetate or succinate as sole carbon sources, and anaerobically with glucose alone (under $H_2 + 5\%$ CO_2). The plates were examined at intervals over a period of 72 hr and the growth patterns of mutant organisms scored. Selected mutants were purified by single colony isolation, and their growth requirements confirmed by further tests on solid and/or in liquid media. Generally, only one mutant of each of the classes emerging was selected from any one culture treated with mutagen and this was numbered accordingly.

Growth tests. Growth tests in liquid media were performed in optically matched ($16 \times 1{\cdot}4$ cm.) ⊥-tubes (Monod, Cohen-Bazire & Cohn, 1951) containing 5 ml. medium rocked at 37° in a water bath at a rate of 30 oscillations/min. and a 10 cm. excursion. The inoculum equivalent to 25 μg. dry wt of organisms was prepared from overnight L-broth cultures washed twice and resuspended in saline. Anaerobic incubation was in similar tubes which were sealed with a rubber bung immediately after mixing a few drops of Na_2CO_3 solution (10 %, w/v) with pyrogallol on an absorbent cotton plug in the neck of the tubes. Growth was assessed with an EEL colorimeter fitted with a neutral-density filter (Evans Electroselenium Ltd., Harlow, Essex); an EEL reading of 10 was equivalent to 0·3 mg. dry wt./ml. All cultures which grew were tested for reversion.

Assay of enzymic activities. Cell-free extracts were prepared from suspensions of organisms (equivalent to 50 mg. dry wt/ml. in 0·4 M-phosphate buffer, pH 7·0), disrupted with an ultrasonic cell disintegrator (M.S.E., 100 W.) for 4 min. at 0°, followed by centrifuging at 23,000 *g* for 10 min. at 2°. Samples of the supernatant fluids containing 3 mg. protein were used for all enzyme assays in 3 ml. reaction mixtures. α-Ketoglutarate dehydrogenase was measured by the method of Amarasingham & Davis (1965) with 3-acetyl-NAD as the electron acceptor. α-Ketoglutarate decarboxylase activity was measured at 420 mμ with ferricyanide as the electron acceptor according to Hager & Kornberg (1961). Pyruvate dehydrogenase and decarboxylase were assayed by precisely the same methods but with pyruvate replacing α-ketoglutarate as the substrate. All activities are recorded in terms of μmoles substrate transformed/mg. protein/hr. Protein was measured according to Lowry, Rosebrough, Farr & Randall (1951).

Mating procedure. Overnight cultures of organisms were diluted into fresh L-broth and grown to exponential phase (3 to 5×10^8 bacteria/ml.) at 37° with gentle shaking. The parental cultures were then mixed in pre-warmed L-broth in a 250 ml. Erlenmeyer flask to give 2×10^8 donor bacteria and 10^7 recipients/ml. in a final volume of 10 ml. After stationary incubation for 60 min. at 37° the conjugation mixtures were

diluted with conjugation buffer pH 7·2 (Fisher, 1957) and plated in duplicate on selective media plus streptomycin.

Interrupted mating. In interrupted mating experiments the log. phase bacteria were mixed as above but at 2×10^7 donor and 4×10^8 recipient/ml. After 5 min. incubation to allow pair formation 1 ml. of the mixture was diluted into 100 ml. glucose minimal medium plus sodium L-aspartate (0·02 %) at 37° in an Erlenmeyer flask. Samples (2 ml.) were withdrawn at intervals into ice-cold bijou bottles and shaken for 1 min. at full speed with a Flask Shaker (B.T.L. Laboratory Centre, Birmingham). Treated samples, and further dilutions where necessary, were plated in duplicate on selective media plus streptomycin. The times of marker entry were determined either by selecting recombinants for a single early marker and subsequent scoring of the inheritance of non-selective markers at various time intervals or, by direct selection of all the relevant recombinant classes. In both cases recombinant formation is expressed as a percentage of the number of donor organisms in the mating mixture at the beginning of the experiment.

Cross-streak method. The cross-streak mating procedure was based upon the method of Berg & Curtiss (1967). Approximately 0·01 ml. of a twice-washed 10-fold-concentrated overnight culture of a recipient organism was applied along the diameter of plates of the appropriate selective media and allowed to dry. Loopfuls of log. phase donor bacteria (2×10^6 approx.) were then streaked directly at right angles across the line of the recipient. After 40 hr incubation recombinants were clearly visible in the tail of the donor streak.

Transduction techniques. The methods and media described by Lennox (1955) and Yanofsky & Lennox (1959) were used for transductions with phage P1kc at multiplicities of infection of 10 to 20 phage per recipient organism. At least two successive lysates were prepared on each strain before use in transductions. Difficulty was experienced in obtaining high-titre lysates by the confluent lysis overlayer technique with some of the slow-growing polyauxotrophic *lip*$^-$ and *suc*$^-$ mutants. Consequently, a few transductions were made with the virulent mutant P1vir1 (Ikeda & Tomizawa 1965) using phage multiplicities of 0·05.

In all the genetic studies recombinant colonies were scored after different periods of incubation, depending mainly on the carbon source of the selective medium, e.g. with glucose and other hexoses 40 to 48 hr; succinate, 50 to 60 hr; acetate 80 to 96 hr. Non-selective marker distributions were determined by replica plating from master plates patched with dilute inocula of recombinant colonies; with *lip* as non-selective marker recombinants were depleted of the factor by plating first with acetate + succinate. When fermentative markers were selective, recombinants were purified by single-colony isolation before replica plating. Supplemented glucose minimal media were not very satisfactory for identifying combinations of *suc*$^{+/-}$ with *lip*$^{+/-}$ or *glt*$^{+/-}$. Consequently, diagnostic tests were made with acetate (with or without a lipoic acid supplement) and succinate (with lipoic acid or glutamate supplements) as substrates as well as appropriately supplemented glucose media.

Chemicals. Amino acids, purines, pyrimidines and vitamins including DL-lipoic acid were purchased from Koch–Light Laboratories Ltd., Colnbrook, Slough, Bucks. The sources of other chemicals were α-keto acids (Sigma), 3-acetyl-NAD (Calbiochem) and streptomycin and penicillin (Glaxo).

RESULTS

Isolation of mutant organisms

Preliminary experiments with the lipoic acid mutant of Vise & Lascelles (1967) indicated that replica plating could be used successfully for the isolation of lipoic acid-requiring mutants only when the colonies to be printed were deprived of the factor by prior growth on a replacement medium containing acetate + succinate. It appears that sufficient of the factor is carried over with bacteria, grown in its presence, to permit colony formation on media without added lipoic acid, due presumably to the relatively large inocula which are transferred by replica plating and the extremely small concentration of lipoic acid required (0·25 mμg./ml. for half-maximal growth). Consequently a procedure was developed in which mutagen-treated cultures were expressed in the presence of lipoic acid but screened first for mutants which responded to a mixture of acetate + succinate (see Methods). In the second phase of the screening procedure where growth on standard media was scored, lipoic acid-requiring mutants (*lip*$^-$) were recognized by good growth on glucose + lipoic acid and the ability to grow anaerobically with glucose alone; they also gave a weak response with lysine + methionine and with succinate supplements, but no growth aerobically with glucose, succinate or acetate alone. These *lip*$^-$ mutants accounted for about 2 % of the total number of mutants tested. About the same proportion of acetate-requiring mutants (*ace*$^-$) of the type studied by Henning & Herz (1964) were found; they grew on acetate-supplemented glucose medium, anaerobically on glucose alone, and aerobically on acetate alone.

However, by far the greatest proportion of mutants isolated were succinate-requirers, and these fell into three main categories. The most frequent type (35 %) grew well with succinate-supplemented glucose and with acetate or succinate alone. Some, though not all, were presumed to lack phosphoenolpyruvate carboxylase because they responded to other tricarboxylic acid cycle intermediates, glutamate and aspartate. Mapping by conjugation with one such mutant showed that the wild-type allele entered 19 min. after *proA* and was 75 % linked with the *metA* or *B* of HfrR4. This corresponds to the *glu* locus (Jacob & Wollman, 1961; Glansdorff, 1965), now termed *ppc*. A related type, representing 20 % of the isolates, responded to a succinate supplement and grew with succinate alone but not with acetate. The third main category of succinate-requiring mutants was characterized by good growth with succinate or lysine + methionine supplements and the ability to grow anaerobically on glucose alone; they would not grow on acetate although most appeared to grow on plates of unsupplemented succinate medium after prolonged incubation. The term *suc*$^-$ will be used only in reference to this latter class of succinate-requirer. They represented approximately 30 % of the mutants and the remainder (10 %) was comprised of a variety of types which exhibited different combinations of growth responses on the test media.

The present work was originally confined to an investigation of the *lip*$^-$ mutants but was subsequently extended to include other mutants which responded to lysine + methionine and grew anaerobically, because of their biochemical relatedness and genetic proximity. Mutations were induced in several parental strains including W1485, T3A58, HfrH and the polyauxotrophic female strains AB1325 and XG3 (Table 1). A total of 36 independently isolated *lip*$^-$ mutants have been examined, 33 were obtained by the method described and a further three were obtained by primary selection for mutants

capable of anaerobic growth on glucose but incapable of aerobic growth on lactate. Some ten of the *lip*⁻ strains were tested for syntrophism by parallel streaking and replica plating methods but none was observed. In addition, eight *suc*⁻ mutants of the *lys*+*met* type have been examined; both *lip*⁻ and *suc*⁻ strains exhibit a marked tendency to revert when large inocula are put on glucose minimal medium. Other types of *lys*+*met* mutant were not recovered by the selective methods for mutant isolation used here.

Properties of lysine + methionine-requiring mutants

Growth tests

The growth curves for two representative lysine + methionine-requiring mutants W1485*lip*2 and W1485*suc*1 are compared with those of the parental strain in Fig. 1. Aerobic growth of W1485*lip*2 did not occur unless supplemented with lipoic acid, and then, the final growth was comparable to that of the wild type on unsupplemented medium. The lipoic acid requirement could be replaced by a mixture of acetate + succinate, although the extent of growth was only half that of the wild-type organism (Fig. 1). Neither acetate nor succinate alone was an effective supplement, although with succinate it was difficult to determine whether slight growth took place prior to the reversion which invariably occurred. Lysine + methionine could also replace lipoic acid but the rate and extent of growth were much lower (Fig. 1), and separately they were without effect. No other mixtures of tricarboxylic acid cycle intermediates or related compounds were found to replace the lipoic acid requirement of these strains. Similarly, only lysine + methionine replaced the succinate requirement of W1485*suc*1 (Fig. 1) and they permitted much better growth than for the *lip*⁻ strain, indicating that lysine + methionine primarily overcome a requirement for succinate rather than for both acetate + succinate. This was confirmed by observing that lysine and/or methionine spare the succinate requirement W1485*lip*2 growing on glucose + acetate but have virtually no sparing effect on the requirement for acetate when succinate is present. Maximum growth of the *suc*⁻ strains with either supplement was always less than for the wild type. However, anaerobically both types of mutant grew as well as the parental strain on unsupplemented medium (Fig. 1).

With succinate or acetate as sole carbon source lipoic acid became an obligate requirement for *lip*⁻ mutants; the addition of lysine + methionine, acetate or succinate was without effect. Similarly, lipoic acid was an obligate requirement for growth on pyruvate, lactate, fumarate, malate and glycollate. By contrast, W1485*suv*1 was found to grow on unsupplemented succinate medium, albeit after a much longer lag than was found with the wild type (Fig. 1). This was also the case for most of the *suc*⁻ strains examined here though some, which were similar in all other respects, did not grow during the 80 hr incubation period. The *suc*⁻ strains would not grow with acetate as sole carbon source or with acetate supplemented with succinate (4 mM or equimolar).

Similar growth patterns were obtained with *lip*⁻ and *suc*⁻ derivatives of AB1325 and XG3 although the extent of growth was less. The basic features of the growth responses described were independent of the salts medium used; however, somewhat higher growth yields were obtained with citrate-containing medium E.

These growth tests confirm and extend the nutritional characterization of the two types of lysine + methionine mutant under investigation and such tests have conclusively established that the *lys*+*met* mutant AT2036 is *suc*⁻ not *lip*⁻.

It should be mentioned that, like Back & Westaway (1962), we have observed that the *lip*$^-$ strains only respond to lysine + methionine when large inocula are used, and on solid medium small single colonies develop after 2 or 3 days but only near the initial streak. Their appearance may depend on cross-feeding of acetate formed by anaerobic metabolism or as the result of traces of lipoic acid present in the region of dense growth. Cross-feeding by acetate and succinate becomes a problem when heavy inocula of *lip*$^-$ or *suc*$^-$ strains are spread on glucose minimal selection plates because any *lip*$^+$ or *suc*$^+$ recombinants (or revertants) excrete acetate and succinate, and as a

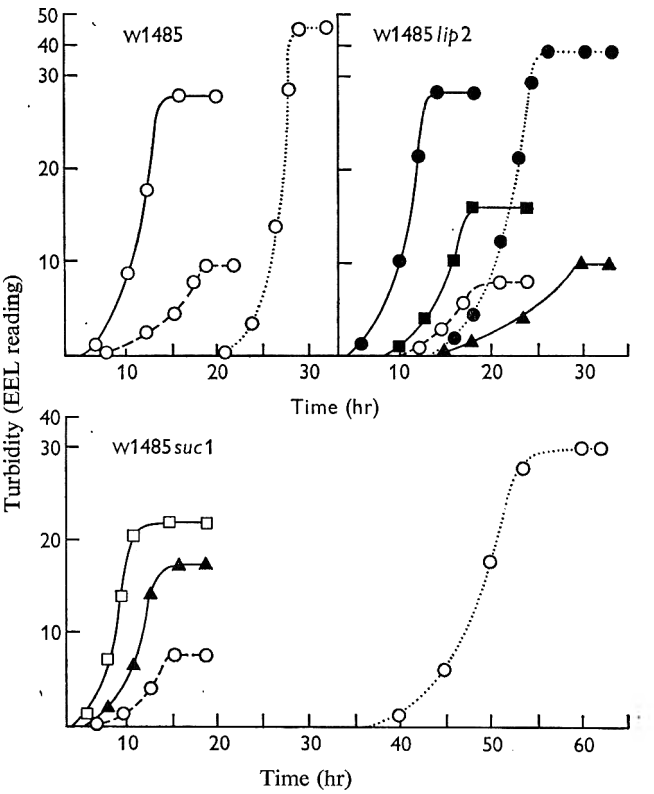

Fig. 1. Growth of *Escherichia coli* W1485, W1485*lip*2 and W1485*suc*1 in different media. Organisms were grown in medium E as described in the Methods section. Incubation was aerobic with glucose (——) and succinate (.....) as carbon source or anaerobic with glucose (– – –). The supplements are indicated thus: ●, lipoic acid: ■, acetate + succinate: □, succinate; ▲, lysine + methionine; and ○, unsupplemented. Growth due to reversion of mutants in unsupplemented media is not shown.

consequence heavy haloes of background growth occur around these colonies and successive generations of revertants develop in the haloes. For this reason, studies involving the selection of *lip*$^+$ or *suc*$^+$ in the presence of their mutants have been performed with succinate and acetate as carbon sources respectively. In neither of these cases does cross-feeding occur.

Enzymic activities

The two lipoic acid-dependent α-keto acid dehydrogenases and their component decarboxylases were assayed in cell-free extracts of *lip*⁻ and *suc*⁺ mutants harvested at the end of the log. phase of aerobic growth on a supplemented glucose minimal medium. Representative results shown in Table 2 indicate that the *lip*⁻ mutants resemble the mutant of Vise & Lascelles (1967) and that the *suc*⁻ mutants lack α-ketoglutarate dehydrogenase activity like the mutant of Hager & Kornberg (1961). Mutant W1485-*lip*2 grown with acetate+succinate supplements lacked pyruvate and α-ketoglutarate dehydrogenase activities although functional decarboxylases were present; dehydrogenase activity was not immediately restored by adding ATP and lipoic acid. However, extracts of organisms grown in the presence of lipoic acid had normal amounts of the enzyme complex. These results are consistent with the primary metabolic lesion being in the biosynthesis of lipoic acid. Extracts of AB1325*lip*13 and XG3*lip*32 gave similar results. A feature common to all the *lip*⁻ strains was a low specific activity of pyruvate decarboxylase in extracts of organisms grown with acetate+succinate as supplement.

No α-ketoglutarate dehydrogenase or decarboxylase activity was detected in extracts of W1485*suc*1, XG3*suc*2 AT2036 (Table 2) and despite differences in the ability to grow on succinate alone, all eight *suc*⁻ strains examined were enzymically similar.

Table 2. *Enzymic activities to extracts of lip⁻ and suc⁻ mutants of Escherichia coli K12*

Organisms were grown aerobically in glucose minimal medium with supplements as shown. Extracts were prepared and enzymes assayed as described in the Methods section.

		Pyruvate*		α-Ketoglutarate*	
Strain	Supplement	pdh	pdc	αkgdh	αkgdc
W1485	None	0·59	1·46	0·15	2·69
W1485*lip*2	Lipoic acid	0·74	1·48	0·25	2·67
W1485*lip*2	Acetate+succinate	–	0·25	–	2·59
W1485*suc*1	Succinate	0·17	1·44	–	–
XG3*suc*2	Succinate	0·47	1·55	–	–
AT2036	Succinate	0·39	1·68	–	–

* The keto acid dehydrogenase and decarboxylase activities are abbreviated as pdh, αkgdh and pdc, αkgdc respectively; –, indicates that no activity could be detected.

Conjugation studies

The foregoing examination of the properties of *lip*⁻ and *suc*⁻ mutants clearly indicate that the *lys+met* mutant AT2036 is *suc*⁻ and not *lip*⁻. As a consequence it was realized that the *lys+met* locus of Taylor & Thoman (1964) must correspond to the *suc*⁻ site, which is also known to map in the same region of the chromosome. The genetic site or sites of the *lip*⁻ mutants were therefore sought by conjugation mapping.

Interrupted mating

In preliminary experiments the gradients of transmission of *proA*, *purB*, *his* and *lip* markers were obtained by conjugation between several Hfr donors (H, C, R4, B11 and AT2572) and AB1325*lip*13. These limited the *lip* site to the region between the origin of HfrC and *purB*.

The *lip*$^-$ site was located more precisely by the interrupted-mating technique with mutants of ABI325 and XG3 as recipients. A typical experiment is shown in Fig. 2 and the entry times obtained from 15 experiments are summarized in Table 3. The values obtained for the standard markers are in reasonable agreement with published results (Curtiss, 1965; Taylor & Thoman, 1964) although the distance between *lac* and *gal* seems to be slightly larger. The *lip* marker entered approximately 2·5 min. after *purE* and 2·9 min. before *gal*. The latter distance suggests that the *lip* site is very close to the original *lys*+*met* site for the *suc*$^-$ mutant, AT2036 (2·75 min. before *gal*). In an attempt to elucidate the relative order of *lip* and *suc*, interrupted matings were performed with

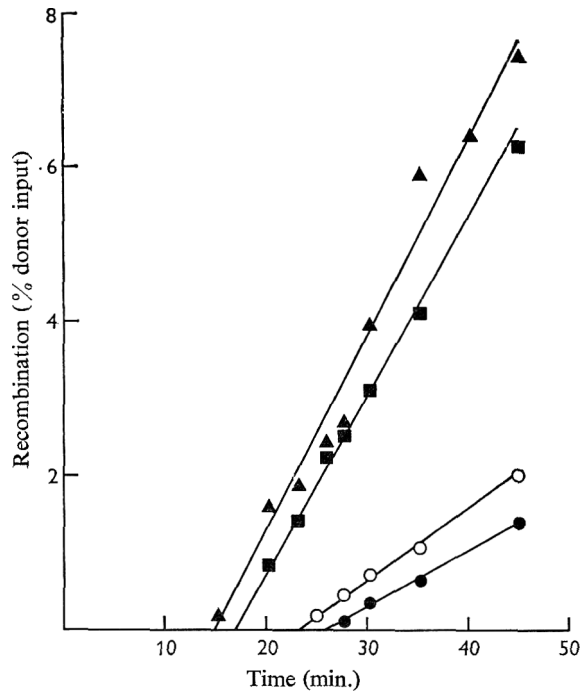

Fig. 2. Time of entry for HfrH markers into *Escherichia coli* ABI325*lip*13. In this experiment *proA*$^+$ recombinants (▲) were selected at different times and subsequently scored for the inheritance of the non-selective markers *lac*$^+$ (■), *lip*$^+$ (○) and *gal*$^+$ (●).

suc$^-$ derivatives of ABI325, XG3 as well as AT2036 (Table 3). With HfrH as donor and AT2036 or ABI325*suc*1 as recipients, distances between *suc* and *gal* were obtained reproducibly which indicated that *lip* and *suc* were within 0·5 min. of each other. However, with *suc*$^-$ derivatives of XG3 the *purE-suc* distance suggested that the two sites (*lip* and *suc*) were separated by more than 2 min. Experiments with HfrB11 and AT2572 as donors supported the latter result (Table 3), and to emphasize the differences crosses with HfrB11 were repeated at 32°. At the lower temperature 1·8 min. separated *suc* and *gal* whereas *lip* and *gal* were separated by 4·8 min.

Linkage analysis

Because of the conflicting results for the positions of *lip* and *suc*, further evidence was sought by examining the linkage relationships between these markers and the

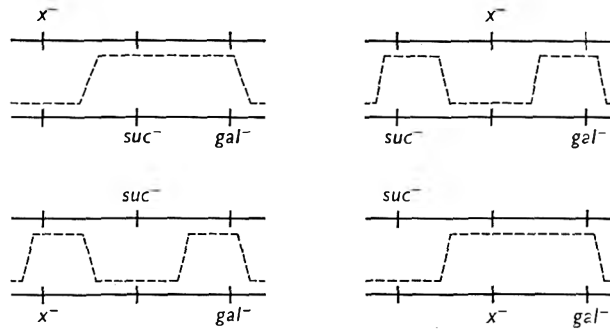

Fig. 3. *Escherichia coli.* Possible arrangements of marker *x* with respect to *suc* and *gal* in reciprocal crosses. The broken lines represent the crossing over necessary for the formation of (*x*$^+$, *suc*$^+$, *gal*$^+$) recombinants.

Table 3. *Times of entry for genetic markers of several Hfr strains*

Donor marker	Time of entry (min.) with* (1) HfrH	(2) HfrH	(3) B11	(4) AT2572	Distance between markers (min.)†
proA$^+$	15·0	.	.	.	2·7
lac$^+$	17·7	.	.	.	0·6
proC$^+$	.	18·3	.	.	2·5
purE$^+$	.	20·8	.	.	2·5
lip$^+$	23·4	23·0	12·5	48·0	2·9
suc$^+$	23·5	25·8	15·0	46·0	
gal$^+$	26·3	.	16·0	.	
purB$^+$	.	.	24·5	.	
his$^+$	.	.	.	8·0	

* The recipients used in columns 1, 3 and 4 were AB1325*lip*9, 13, AB1325*suc*1 and AT2036. In column 2, XG3*lip*31, 32 and XG3*suc*6 and 8 were the recipients.

† The distance between markers is an average taking into account all of the values obtained with HfrH.

gal and *purE* loci. The linkages observed in a number of crosses were: *gal–lip* (46–68 %), *gal–suc* (78–90 %), *lip–purE* (77 %) and *suc–purE* (67 %), which suggest the order *purE–lip–suc–gal*. The selection of *gal*$^+$ recombinants in the presence of *lip*$^-$, and to a lesser extent *suc*$^-$, leads to very poor growth on the *gal*$^+$ selective medium and there may be some preferential selection of the wild type alleles. The suspected order was confirmed by three factor crosses of the type illustrated in Fig. 3. If marker *x* represents *lip* and the order is *lip–suc–gal* then (*lip*$^+$, *suc*$^+$, *gal*$^+$) recombinants would be the result of double crossing-over when *lip*$^-$ is a donor marker, and quadruple crossing-over when *lip*$^-$ is a recipient marker. If the order is *suc–lip–gal*, opposite predictions would be made. Thus, by comparing the ratio of (*lip*$^+$, *suc*$^+$, *gal*$^+$) to *gal*$^+$ recombinants in a reciprocal cross, the highest ratio should be produced by the order which depends on double crossing-over. The results of such crosses are given in Table 4. The frequency of (*lip*$^+$, *suc*$^+$, *gal*$^+$) recombinants relative to the selected *gal*$^+$ is clearly highest when *lip*$^-$ is the donor allele and this is only consistent with the order *lip–suc–gal*.

The three factor crosses yielded (*lip*$^-$, *suc*$^-$) double mutants which were characterized

by their inability to grow with acetate, acetate plus lipoic acid, succinate, glucose or glucose supplemented with lipoic acid, but the ability to respond to glucose supplemented with lipoic acid + succinate and succinate medium supplemented with lipoic acid.

Table 4. *The order of lip and suc markers with respect to gal*

Mating mixtures (10^7 donors and 2×10^8 recipients/ml.) were incubated for 60 min. at 37° and *gal*$^+$ recombinants selected on enriched streptomycin minimal medium supplemented with lipoic acid and succinate. Recombinants were purified and the distribution of non-selective markers was determined with a variety of diagnostic media. Counter selection for *lac*$^-$ had no effect on the linkage relationships and is not recorded.

Cross		*Gal*$^+$ recipients		Distribution of non-selective markers		
Donor	Recipient	Total/ml.	Tested	Classes	No.	Frequency
HfrH*lip*22 (*gal*$^+$, *lip*$^-$, *suc*$^+$)	ABI325*suc*1 (*gal*$^-$, *lip*$^+$, *suc*$^-$)	21,000	196	*lip*$^+$, *suc*$^+$	85	43·4%
				lip$^+$, *suc*$^-$	19	9·7%
				lip$^-$, *suc*$^+$	89	45·4%
				lip$^-$, *suc*$^-$	3	1·5%
HfrH*suc*3 (*gal*$^+$, *lip*$^+$, *suc*$^-$)	ABI325*lip*9 (*gal*$^-$, *lip*$^-$, *suc*$^+$)	5,700	80	*lip*$^-$, *suc*$^+$	5	6·3%
				lip$^-$, *suc*$^-$	43	53·7%
				lip$^-$, *suc*$^+$	12	15·0%
				lip$^-$, *suc*$^-$	20	20·5%

Table 5. *Transfer of genetic markers by F′-donors*

Matings were performed by the cross-streak technique (see Methods) using appropriate selective media. Selection against the donor was normally with both streptomycin and donor nutritional requirements but with streptomycin-sensitive recipients only the latter selection was possible (except for the prototrophic donors ORF-1 and W3101). Controls with Hfr and F$^+$ donors were also included.

F′-donor*	Transfer of donor markers					
	lac$^+$	*proC*$^+$	*purE*$^+$	*lip*$^+$	*suc*$^+$	*gal*$^+$
ORF-1	+	+	+	−	−	−
ORF-4	+	+	+	+	−	−
ORF-8	+	+	+	−	−	−
ORF-15	+	+	+	−	−	−
W3101(F_2-*gal*)	tr	tr	tr	+	+	+

* See Table 1 for details of donor strains.

Experiments with F′ donors

The genetic sites of the five *lip*$^-$ strains examined by conjugation were located in the same region of the chromosome, and a rapid method was sought for testing the remaining mutants. Using the cross-streak technique, five F′-donors (four primary or haploid and one secondary or partial diploid) were mated with all the *lip*$^-$ mutants and several *suc*$^-$ mutants on plates of selective medium. The method was successful with F$^+$ and Hfr strains as recipients as well as with F$^-$ strains, if nutritional selection against the donor were possible. The markers transferred are summarized in Table 5, and of the primary F′-donors, ORF-4 alone transferred the *lip* marker with *purE*, *proC* and *lac* but not *suc*. Clearly, the *lip* locus is closer to *purE* than the *suc* locus and the chromosomal segment incorporated by the F′ factor of ORF-4 extends to a point between the *lip* and *suc* sites, whereas the other primary F′ factors fall short of the *lip* site. With the secondary F′, W3101(F_2-*gal*). both *lip* and *suc* were transferred in addi-

tion to *gal*, and a relatively poor transfer of *lac*, *proC* and *purE* markers was also observed (Table 5). The latter was assumed to be due to chromosomal mobilization by the episome. All 36 *lip*⁻ mutants gave identical results in these tests, indicating that, if they do not represent functionally identical sites, then the corresponding genes are closely linked.

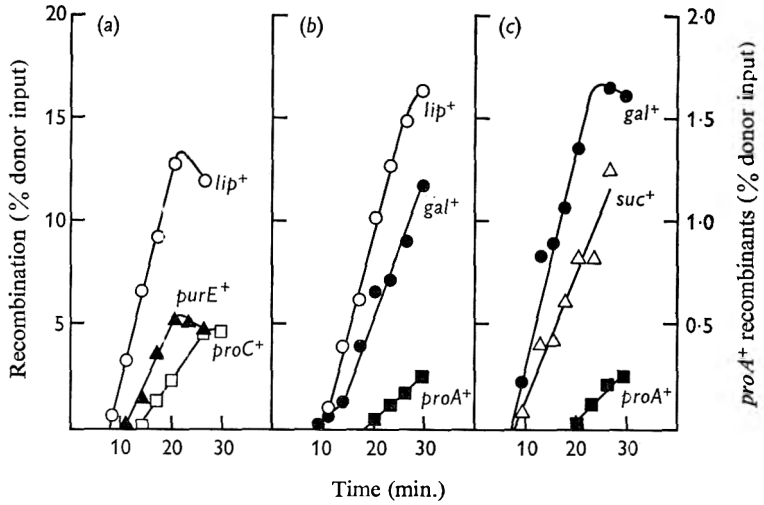

Fig. 4. *Escherichia coli*. Results of interrupted mating experiments between F′ donors and *lip*⁻ and *suc*⁻ mutants, (*a*) ORF-4 × XG3*lip*32; (*b*) W3101(F_2-*gal*) × ABI325*lip*9, and (*c*) W3101-(F_2-*gal*) × ABI325*suc*1. This mating procedure was as described in the Methods and all recombinants were selected by direct plating. Entry of *proA*⁺ at a lower frequency was due to chromosome mobilization.

The F′ donors were also used in interrupted mating exeriments with several *lip*⁻ and *suc*⁻ mutants. In accordance with the polarity assigned to ORF-4 (Berg & Curtiss, 1967), the *lip*⁺ marker was first to enter at 8·0 min., followed by *purE*⁺ at 10·8 min. and *proC*⁺ at 13·9 min. with XG3*lip*32 (Fig. 4*a*). In similar experiments W3101(F_2-*gal*) was mated with ABI325*lip*9 (Fig. 4*b*) and ABI325*suc*1 (Fig. 4*c*). With this donor *gal*⁺ was transferred first and *suc*⁺ entered 0·5 min. after *gal*⁺, *lip*⁺ 2 min. after *gal*⁺, and finally, at a much lower frequency, *proA*⁺ entered by chromosome mobilization 11 min. after *gal*⁺. The problem of selecting *gal*⁺ when *lip* is non-selective is illustrated in Fig. 4*b* where the recovery of *gal*⁺ recombinants is poor until *lip*⁺ has entered. Nevertheless, the results are consistent with the relative order of markers *purE–lip–suc–gal*.

Transduction studies

As soon as the early conjugation studies had indicated that the mutant loci of *lip*⁻ and *suc*⁻ derivatives of ABI325 were closely linked, parallel transduction studies using phage P1kc were initiated, to determine whether these sites are cotransducible with each other or with other markers in the same region of the chromosome. Transduction of *lip*⁻ and *suc*⁻ mutants to prototrophy was feasible when succinate and acetate were employed as the selective media respectively (Table 6); interference from revertants generally precluded the use of a glucose medium. However, only the time required for colony development was affected by selecting *his*⁺, *trp*⁺ or *lip*⁺ transductants on glu-

cose, succinate or acetate media, and direct comparison of the transduction frequencies for different markers with different substrates was therefore considered valid. With *lip*⁻ recipients high transduction frequencies were obtained, the frequency for the *lip* marker being approximately the same as for *trp* and twice that of *his* (Table 6). By contrast, transduction frequencies for all markers were low with *suc*⁻ recipients (Table 6); transduction in the *suc* region being as frequent as for *his* but only half as frequent relative to *trp*. From these results it can be deduced that the relative transduction frequency *lip*⁺/*suc*⁺ is 2·02 to 2·16 and the ratio *trp*⁺/*his*⁺ is 1·96 to 2·10, which corresponds to the value reported by Yanofsky & Lennox (1959). Abortive transductants were present in all cases.

Table 6. *Relative frequencies of transduction in the lip⁻ and suc⁻ regions*

In all experiments P1kc lysates prepared on the prototrophic donor strain W1485 were used to infect the recipients (5×10^8/ml.) at a multiplicity of 20. *Lip*⁺ recombinants were selected on succinate medium, *suc*⁺ recombinants on acetate medium and the *his*⁺ and *trp*⁺ recombinants were selected on glucose medium. Other mutants examined in identical experiments were: AB1325*lip*13, 18; XG3*lip*31; T3A58*lip*26 and XG3*suc*7, 9, 11; the results with these are included in the average values for the transduction frequencies and the total number of determinations is given in parenthesis.

Recipients		Transductants (10^3/ml.)				Frequency relative to			
		lip⁺	*suc*⁺	*his*⁺	*trp*⁺*	*his*⁺	(Avge)	*trp*⁺	Avge
W1485*lip*2	(*lip*⁻)	17·9	.	.	.	.	.	.	.
AB1325*lip*9	(*lip*⁻, *his*⁻)	59·1	.	29·2	.	2·02	2·12 (15)	.	.
XG3*lip*32	(*lip*⁻, *trp*⁻)	112·8	.	.	92·1	.	.	1·22	1·08 (12)
T3A58*lip*25	(*lip*⁻, *trp*⁻)	57·7	.	.	55·7	.	.	1·04	
W1485*suc*1	(*suc*⁻)	.	3·61	.	.	.	.	.	.
AT2036	(*suc*⁻, *his*⁻)	.	5·40	5·15	.	1·05	1·05 (11)	.	.
AB1325*suc*1	(*suc*⁻, *his*⁻)	.	3·87	3·81	.	1·02		.	.
XG3*suc*8	(*suc*⁻, *trp*⁻)	.	7·20	.	14·6	.	.	0·49	0·50 (7)

* In the case of T3A58 the transductants are *trpA*⁺.

Table 7. *Tests for cotransduction of lip and suc with other markers*

Linkage between *lip* or *suc* and the other markers was examined by transduction with phage P1kc in which *lip* or *suc* and the other markers were selective. Selection was on appropriately supplemented media with the following substrates: *lip*⁺, succinate; *suc*⁺ and *glt*⁺, acetate; other markers, glucose. In the case of *suc* and *gal* the frequency of cotransduction was different if (*a*) *suc*⁺ or (*b*) *gal*⁺ was selective.

Markers	Linkage (%) with *lip*	Linkage (%) with *suc*
lac	< 0·01	< 0·35
proC	< 0·23	< 0·72
T_6^s	< 0·23	< 0·72
purE	< 0·23	< 0·72
glt	< 0·63	—
gal	< 0·01	(*a*) 36·1
		(*b*) 52·4

Tests for cotransduction of lip and suc with other markers

Despite numerous tests with five *lip*⁻ mutants as both donors and recipients, no linkage was ever observed between *lip* and any of the other markers tested (Table 7). In the case of the *suc*⁻ mutants, cotransduction with *glt* (see below) and *gal* was ob-

served (Table 7). When *suc*$^+$ was selective and *gal*$^{+/-}$ non-selective, the average linkage for a total of 15 determinations with 7 mutants was 36·1 % and different mutants gave values ranging from 31 % to 42 %. However, closer linkage was observed with *gal*$^+$ as selective marker; seven determinations with two mutants gave average values of 51·8 % and 54 %. Similar observations have been made by U. Henning (personal communication). Cotransduction of *suc* with *gal* has also provided a successful method for determining the order of several *suc*$^-$ loci relative to *gal*.

The question of whether *lip* and *suc* are cotransducible presented a number of technical difficulties, the principal ones being the problem of selecting *lip*$^+$ in the presence of *suc*$^-$ and the general uncertainty of having to base linkage on the inheritance of markers (*lip*$^-$ and *suc*$^-$) which are known to be relatively unstable. Nevertheless, selection of *suc*$^+$ transductants on acetate medium plus lipoic acid from crosses between *lip*$^-$ donors and *suc*$^-$ recipients indicated that *suc* and *lip* are not cotransducible (< 0·5 % linkage). Also, by making use of the ability of *suc*$^-$ to grow with succinate as substrate, *lip*$^+$ transductants were selected from crosses between AB1325-*suc*1 and AB1325*lip*9 but again no linkage (< 0·9 %) was observed. Finally, convincing evidence for the lack of linkage between *lip* and *suc* came from experiments made possible by the isolation of (*lip*$^-$, *suc*$^-$) double mutants from the conjugation experiments reported above (see Table 4). Using two such double mutants as recipients and a wild-type donor, *lip*$^+$, *suc*$^+$ and (*lip*$^+$, *suc*$^+$) transductants were selected. All the *lip*$^+$ transductants tested were *suc*$^-$, all the *suc*$^+$ transductants were *lip*$^-$ and no (*lip*$^+$, *suc*$^+$) recombinants were ever obtained by direct selection on unsupplemented acetate medium. In all these crosses normal frequencies of *lip*$^+$ and *suc*$^+$ transductants were obtained relative to *his*$^+$. The results of these transduction studies confirm that the *lip* and *suc* loci are farther apart than was indicated by the first interrupted mating experiments.

The position of the glt locus relative to lip and suc

Another gene which maps in the region of the *Escherichia coli* chromosome under investigation is the citrate synthase gene or *glt* locus (Ashworth, Kornberg & Nothmann, 1965). Transduction crosses between two *glt*$^-$ mutants and several *lip*$^-$ mutants gave no evidence for linkage whether *lip*$^+$ or *glt*$^+$ was selective (Table 7). However, in crosses between W620 (*glt*$^-$, *gal*$^-$) and wild type, cotransduction, of *glt* with *gal* was observed; 41 % to 49 % linkage when *glt*$^+$ was selective and 45 % to 58 % with *gal*$^+$ selective. Slightly less linkage was found with W1485*glt*$^-$, 19 % to 24 % (*glt*$^+$ selective) and 22 % to 30 % (*gal*$^+$ selective). These values indicate that the *suc* and *glt* loci are closely linked. Since no satisfactory method is available for direct selection of *suc*$^+$ or *glt*$^+$ in the presence of the alternate mutant alleles, two indirect approaches were adopted for further investigation of the linkage between them. The first consisted of transduction crosses of the type illustrated in Fig. 3, where *x* represents *glt*. Accordingly, non-selective marker distribution was scored for *gal*$^+$ recombinants obtained in crosses between *suc*$^-$ and *glt*$^-$ mutants (Table 8). By assuming that the least-frequent class arises from quadruple exchanges rather than from double exchanges the results indicate that the gene order must be *glt–suc–gal*. These results also show a *glt–suc* linkage of approximately 90 %. In the second approach (*suc*$^+$, *glt*$^+$) transductants were selected directly on acetate medium from crosses in which *gal* was used as an outside marker (Table 9). The distribution of the donor *gal* marker in these transductants was

normal with *suc*$^+$ donors but much reduced when the donor was *glt*$^+$, and this is consistent only with the marker order *glt–suc–gal*. Again, very close linkage between *glt* and *suc* was indicated and a map distance of 7·1 relative to *his* can be calculated from the results of cross 3 (Table 9).

Table 8. *Order of the suc and glt loci relative to gal*

Transductions were with phage P1vir1 (see Methods); *gal*$^+$ recombinants were selected in the presence of glutamate and succinate and subsequently scored for the inheritance of *suc* and *glt* alleles using a variety of test media.

P1vir1 donor	Recipient	Selective marker	No. of transductants per ml.	No. scored	Percentage of *gal*$^+$ transductants scored as (*glt*$^+$, *suc*$^+$)	(*glt*$^+$, *suc*$^-$)	(*glt*$^-$, *suc*$^+$)	(*glt*$^-$, *suc*$^-$)
W1485*glt*$^-$ (*glt*$^-$, *suc*$^+$, *gal*$^+$)	AT2036 (*glt*$^+$, *suc*$^-$, *gal*$^-$)	*gal*$^+$	170	95	4	45	51	0
W1485*suc1* (*glt*$^+$, *suc*$^-$, *gal*$^+$)	W620 (*glt*$^-$, *suc*$^+$, *gal*$^-$)	*gal*$^+$	290	90	3	43	47	7

Table 9. *Outside-marker distribution in (glt*$^+$, *suc*$^+$) *transductants*

Transduction crosses were performed with phage P1kc as described in the Methods section, and (*glt*$^+$, *suc*$^+$) recombinants were selected on acetate media and subsequently scored for the inheritance of *gal*. *His*$^+$ recombinants were selected on glucose media supplemented with succinate and glutamate.

Cross	P1kc donor	Recipient	Transductants: Type	No./ml.	No. scored	Distribution of non-selective marker of donor: Marker	Percentage
1	W1485*suc1* (*suc*$^-$, *gal*$^+$)	W620 (*glt*$^-$, *gal*$^-$)	(*glt*$^+$, *suc*$^+$)	1620	128	*gal*$^+$	4·7
2	W620 (*glt*$^-$, *gal*$^-$)	W1485*suc1* (*suc*$^-$, *gal*$^+$)	(*glt*$^+$, *suc*$^+$)	108	54	*gal*$^-$	32·0
3	W1485*glt*$^-$ (*glt*$^-$, *gal*$^+$)	AT2036 (*suc*$^-$, *gal*$^-$)	(*glt*$^+$, *suc*$^+$) *his*$^+$	120 1680	66	*gal*$^+$	34·0

DISCUSSION

Of the many mutants of *Escherichia coli* obtained by screening for response to acetate and/or succinate two classes also responded to lysine + methionine. An investigation of the nutritional characteristics and enzymic constitution of these mutants was clearly consistent with their representing: (*a*) one class of succinate-requirer (*suc*$^-$) lacking α-ketoglutarate dehydrogenase; (*b*) mutants which require acetate plus succinate due to a primary lesion in the biosynthesis of lipoic acid (*lip*$^-$). The replacement of succinate by lysine + methionine for *suc*$^-$ mutants can be explained in part by the participation of succinylCoA in the biosynthesis of these amino acids. However, it is difficult to understand how they obtain succinylCoA for porphyrin synthesis. The same problem applies to the growth of *lip*$^-$ mutants with lysine + methionine and in addition it is necessary to explain how these mutants meet the demand for acetylCoA under these conditions. Vise & Lascelles (1967) suggested that the pyruvate-induced pyruvate oxidase (Gounaris & Hager, 1961) may supply sufficient acetate when lysine and methionine are present. It should also be emphasized

that the growth of *lip*⁻ mutants with lysine + methionine is extremely poor and large inocula are essential. As a consequence of these nutritional and enzymological investigations it was realized that the *lys* + *met* and *suc* sites on the linkage map of *Escherichia coli* represent identical mutant loci; this has been acknowledged by Taylor & Trotter (1967) in their recently revised linkage map; the *lys* + *met* terminology has now been dropped.

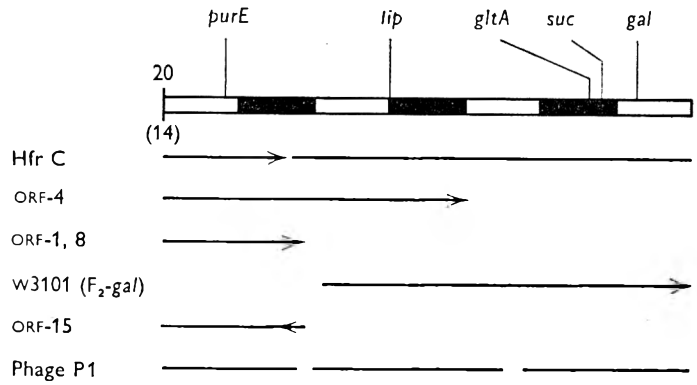

Fig. 5. A section of the linkage map of *Escherichia coli*, showing the relative order and positions of the loci examined here. The map is marked in 1 min. divisions commencing at 20 min. relative to the initiation of mating with HfrH or 14 min. according to Taylor & Trotter (1967). The arrowheads indicate the direction of transfer of Hfr and F′ donors; the extremities of the fragments transferred by F or phage P1 are not specified within the intergenic regions.

The genetic studies reported here are summarized in Fig. 5 and it can be seen that the *lip* and *suc* sites are located in the same segment of the *Escherichia loci* chromosome. It is not understood why mapping by interrupted mating with HfrH and AB1325*suc*⁻ or *lip*⁻ strains consistently indicates that the two sites are extremely close, when other conjugation and transduction studies suggest that they are separated by at least 2 min.

No genetic or nutritional evidence for the presence of more than one class of *lip*⁻ mutant was obtained with the 36 strains examined here, even though it would be reasonable to assume more than one metabolic reaction is unique to the biosynthesis of lipoic acid. However, if the genes controlling lipoic acid biosynthesis are clustered (as has recently been found for the genes responsible for biotin biosynthesis; del Campillo-Campbell, Kayajanian, Campbell & Adhya, 1967) the *lip*⁻ mutants could still correspond to defects in several *lip* genes. The absence of cross-feeding may be due to non-permeating intermediates which might be anticipated if lipoic acid is synthesized at the thioester level, e.g. from octanoylCoA, or if synthesis is completed subsequent to the binding of a precursor to the dehydrogenase complex.

The 8 *suc*⁻ mutants examined in detail also fall into a single category: they are all defective in the decarboxylase component of the α-ketoglutarate dehydrogenase complex. Isolation of a second class of *suc*⁻ mutant lacking the lipoamide transsuccinylase component would have been anticipated, by analogy with pyruvate dehydrogenase where the corresponding decarboxylase (*aceE*) and transacetylase (*aceF*) mutants have been isolated. However the *aceF* mutants constitute a minority class of 19 out of 109 *ace*⁻ strains examined (Henning, Dietrich & Deppe, 1968). Further

suc⁻ strains are being screened for mutants lacking the transsuccinylase. Since the dihydrolipoamide dehydrogenase components of pyruvate and α-ketoglutarate dehydrogenases are interchangeable *in vitro*, the failure to isolate *ace*⁻ and *suc*⁻ mutants lacking this activity is taken to indicate that there are two genes responsible for the corresponding components. When two mutants responding to acetate + succinate but not lipoic acid were isolated, it was immediately thought they might be defective in the gene for a common dihydrolipoamide dehydrogenase or some other common structural or regulatory component of the dehydrogenase complexes. Alternatively, they could represent mutants blocked in the lipoic acid-activating system necessary for the binding of lipoic acid to the enzyme complex (Reed, Leach & Koike, 1958). However, upon further examination they proved to be double mutants of the (*ace*⁻, *suc*⁻) type. The *Aerobacter aerogenes* lysine + methionine mutant 19, which, like the *Escherichia coli suc*⁻ strains, lacks α-ketoglutarate decarboxylase activity, differs in its inability to grow with succinate as sole carbon source. This is probably not crucial because the *suc*⁻ strains of *E. coli* gave different responses with succinate as sole carbon source, so this property may not be directly related to the lack of α-ketoglutarate dehydrogenase.

It may be significant that the genes for two enzymes of the tricarboxylic acid cycle, citrate synthase and α-ketoglutarate dehydrogenase, map in the same region of the chromosome. Moreover, in view of the high degree of linkage between the two sites it is conceivable that they are adjacent. The relative order of the *glt* (citrate synthase) and *suc* loci established here by transduction (Fig. 5) agrees with the findings of E. L. Wollman (personal communication). The citrate synthase gene has recently been designated *gltA* by Taylor & Trotter (1967).

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Stimulation of Germination of Bacterial Spores by Analogues of D-Alanine

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SUMMARY

L-Alanine-initiated germination of spores of *Bacillus cereus* was potentiated by the following structural analogues of alanine: *O*-carbamyl-D-serine (OCDS), D-cycloserine (DCS), *β*-alanylhydroxamic acid (*β*AHA) and glycyl hydroxamic acid (GHA); but not by D-*α*-alanyl-hydroxamic acid (DAHA). Potentiation of germination resulted from inhibition of alanine racemase in the spores with consequent suppression of the formation of D-alanine, an inhibitor of L-alanine-initiated germination. OCDS was the most effective potentiator of germination and inhibitor of racemase. *β*AHA and GHA were more effective potentiators of germination than could be explained solely by their weak inhibition of the racemase. The extra effectiveness was associated with slow binding of the analogues to the spores, and possibly also with formation of hydroxylamine which also inhibited alanine racemase and potentiated L-alanine-initiated germination. Germination initiated by ribosides and amino acids other than L-alanine was not strongly potentiated by OCDS, arguing against a role for L-alanine as an intermediate. However, germination initiated by adenosine + D-alanine was strongly inhibited by OCDS, which argues for the role of D-alanine being to supply the L-isomer by racemization. Antibacterial activities of the analogues did not mirror their activities as potentiators of germination.

INTRODUCTION

Since the pioneering work of Hills (1949, 1950) the importance of L-alanine as an initiator and D-alanine as an inhibitor of germination of many types of bacterial spores has been amply confirmed. Inhibition of L-alanine-initiated spore germination by D-alanine (Hills, 1949) probably occurs because the D-isomer inhibits the enzyme which handles L-alanine, important in germination. This enzyme may be L-alanine dehydrogenase (O'Connor & Halvorson, 1961*a*, *b*) or some other as yet unidentified enzyme (Freeze & Cashel, 1965). That inhibitory D-alanine could be formed from L-alanine by the spores themselves was shown by the demonstration of alanine racemase activity in spores of *Bacillus cereus* T and other organisms (Stewart & Halvorson, 1953). Church, Halvorson & Halvorson (1954) showed that spore germination and racemase activity could be dissociated from each other, for instance by change of pH value; racemase in spores therefore appeared normally to hinder rather than play a positive role in L-alanine-initiated germination, particularly with concentrated spore suspensions (Fey, Gould & Hitchins, 1964). The magnitude of the hindrance was emphasized by Krask (1961), who showed that D-cysteine stimulated L-alanine-initiated

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germination of *B. cereus* T spores by inhibiting alanine racemase and thereby preventing formation of D-alanine, and by Gould (1966), who showed that two racemase inhibitors (D-cycloserine and *O*-carbamyl-D-serine) which are structural analogues of D-alanine stimulated L-alanine-initiated germination.

Substances which stimulate germination (and therefore heat- and radiation-sensitization) of spores might find useful application as spore-control agents in materials in which spores are normally present but unwanted (e.g. in most foodstuffs). We have therefore studied the action, on spore germination and on the activity of spore alanine racemase, of a number of alanine analogues, including optically inactive and D-stereroisomers, with L-alanine and other chemical agents as germinants.

METHODS

Spores. Spores of *Bacillus cereus* strain T were used for most experiments. Other organisms used were *B. cereus* PX (Hitchins, Gould & Hurst, 1963); *B. cereus* NCTC 945 and *B. megaterium* NCTC 7851 (National Collection of Type Cultures, Colindale); *B. subtilis* A (rough strain from F. F. Busta; Edwards, Busta & Speck, 1965); *B. subtilis* syn. *globigii* (Fey *et al.* 1964); *B. pumilus* S 3, and *B. polymyxa* M 1 (laboratory isolates). Spores were grown on potato yeast-extract glucose agar at 37° as described by Hitchins *et al.* (1963). When sporulation and lysis of sporangia was complete the spores were scraped from the agar, washed six times with cold distilled water by centrifugation and stored at 4° in water at a concentration equivalent to about 20 mg. dry weight/ml. When heat-activated spores were required, samples of suspensions were heated at 70° for 30 min. immediately before use.

Media and measurement of germination. Germination media normally contained sodium phosphate (80 mM, pH 8·0). The buffer was sodium acetate (80 mM, pH 8·0) whenever calcium dipicolinate was used. Germinants and related compounds used (Koch–Light Laboratories Ltd., Colnbrook, Bucks) were L-alanine, D-alanine, L-cysteine (all 10 mM); inosine (0·2–1·0 mM); adenosine (1 mM); adenosine (1 mM for unheated, 100 μM for heat-activated spores) + L-alanine or other amino acids (10 and 100 μM for unheated and heat-activated spores respectively); *n*-dodecylamine (1/5 saturated soution): calcium dipicolinate (40 mM) was made by mixing sodium dipicolinate solution (made from sodium hydroxide and dipicolinic acid; Aldrich Chemical Co. Inc., Milwaukee 10, Wisconsin) and calcium chloride solution immediately before use in the sodium acetate buffer.

Germination was measured by mixing spores with the media at 37° and reading extinction of the suspensions at intervals with an absorptiometer ('Biochem', Hilger and Watts Ltd., Camden Road, London). Germination was accompanied by a decrease in extinction. Phase-contrast microscopy was used to confirm germination: ungerminated spores were phase-bright and germinated spores phase-dark.

Alanine analogues. Analogues of the D-isomer used were: D-cycloserine (Oxamycin; D-4-amino-3-isoxazolidone), which was a gift from Eli Lilly and Co. Ltd. (Basingstoke, Hants); *O*-carbamyl-D-serine, a gift from Dr H. P. Hidy (Commercial Solvents Corp., Terre Haute, Indiana); D-α-alanyl hydroxamic acid (synthesized by Mr A. P. Rhodes of this Laboratory). Optically inactive analogues of alanine used were glycyl hydroxamic acid and β-alanyl hydroxamic acid (Hynes Chemical Research Corp., Durham, North Carolina). Similarities in structure of the analogues are indicated in Fig. 1.

The analogues were dissolved in water and mixed with warmed germination media before use as indicated in Results.

Growth-inhibitory concentrations of alanine analogues. The analogues were sterilized by filtration through sintered glass (grade 11/5) and incorporated in nutrient broth at 25 mM, and then doubling dilutions were made down to 0·195 mM by using as diluent broth inoculated with 5% (v/v) overnight broth cultures of one of the following organisms: *Escherichia coli* NCIB 9483 (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen); *Pseudomonas aeruginosa*; *Streptococcus faecalis* (laboratory isolates); *Staphylococcus aureus* OXFORD (NCTC 6571); *Bacillus cereus* T. The cultures were then incubated at 37° for 24 hr and the minimum concentrations of analogues inhibiting growth were recorded.

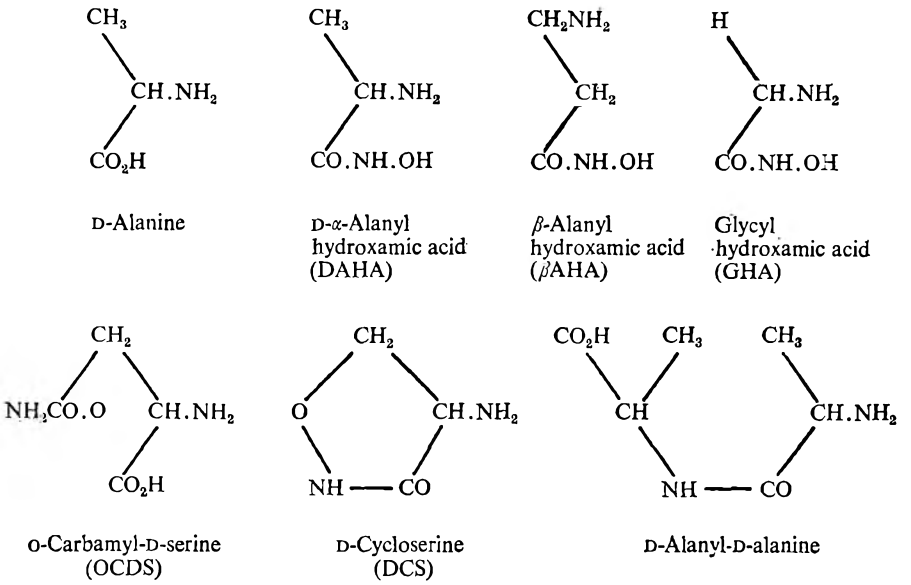

Fig. 1. Formulae of the alanine analogues studied and of D-alanine and D-alanyl-alanine for comparison. The formulae are written to show similarities of structure.

Alanine racemase. Racemase activity of intact spores was determined by using L-alanine as substrate and measuring the rate of production of D-alanine as described by Fey *et al.* (1964) with D-amino acid oxidase (from hog kidney, British Drug Houses Ltd., Poole, Dorset; Wood & Gunsalus, 1951). Incubation mixtures at 37° contained spores (equiv. 1 mg. dry weight/ml.), L-alanine (100 mM) ± analogues, and sodium phosphate (80 mM, pH 8·0). At 10 min. intervals up to 60 min., samples (1·5 ml.) were removed, cooled in ice, and centrifuged in the cold. D-Alanine formed by racemization was then estimated by incubating samples of the supernatant fluid (0·5 ml.) with D-amino acid oxidase (15 mg.) and sodium phosphate (80 mM, pH 8·0) in a Warburg apparatus at 30° and measuring the oxygen uptake for 90 min. Under these conditions > 90% oxidation of the D-alanine occurred. It was determined separately that none of the analogues used inhibited D-amino acid oxidase nor were they utilized by this enzyme as substrates at the concentrations at which they might be carried over into the Warburg flasks. Inhibition of alanine racemase by analogues is recorded by expressing activity as a percentage of activity of analogue-free controls.

RESULTS

Effect of analogues on germination initiated by L-*alanine*

Unheated spores of *Bacillus cereus* T germinated very slowly in L-alanine. The rate of germination was decreased by D-α-alanyl hydroxamic acid (DAHA), but the other analogues caused increases in the rates of germination (Fig. 2) in order of increasing activity: β-alanyl hydroxamic acid (βAHA), D-cycloserine (DCS), glycyl hydroxamic acid (GHA) and *O*-carbamyl-D-serine (OCDS). It was noticeable that the pattern of stimulation of germination by DCS differed from that of the other analogues, showing an increase in rate with time (Fig. 2). Figure 3 shows the result of a similar experiment using heat-activated spores in place of the unheated spores used for Fig. 2. The heated

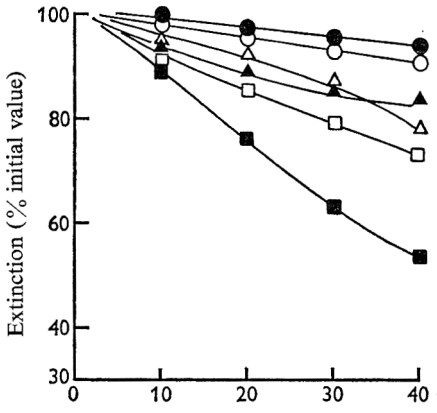

Fig. 2

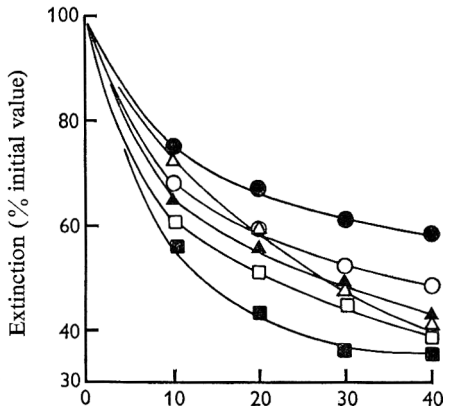

Fig. 3

Fig. 2. Stimulation of germination of unheated spores of *Bacillus cereus* T by alanine analogues. All tubes contained L-alanine (10 mM) in sodium phosphate buffer (80 mM, pH 8·0) and were incubated at 37°. Analogues, used at 10 mM except for *O*-carbamyl-D-serine (6 mM) were: ○, no analogue added (control); ●, D-alanyl hydroxamic acid; △, D-cycloserine; ▲, β-alanyl hydroxamic acid; □, glycyl hydroxamic acid; ■ *O*-carbamyl-D-serine.

Fig. 3. Stimulation of germination of heat-activated spores of *Bacillus cereus* T by alanine analogues. Experimental details and symbols as for Fig. 2 except that the spores were heated for 30 min. immediately before use.

B. cereus T spores germinated more rapidly than the unheated ones, as expected. DAHA again decreased the rate of germination but the other analogues increased the rate of germination, and the late stimulation by DCS was marked. Different rates of germination, but with the same patterns of response, were found with higher and lower concentrations of the analogues and of L-alanine.

Effect of the analogues on germination initiated by germinants other than L-*alanine*

Table 1 summarizes the effects of some of the alanine analogues on germination of spores initiated by the metabolizable germinants inosine, adenosine and L-cysteine and by the non-metabolizable germinants *n*-dodecylamine and calcium dipicolinate (CaDPA). OCDS, the strongest potentiator of alanine-initiated germination, did not

increase the rate of germination of spores with any of these germinants, strongly suggesting that alanine racemase activity is of little importance in governing germination rate in these systems. DCS inhibited germination of unheated spores in inosine but caused an increase in the initial rate of germination of heat-activated spores. Of the four analogues, only DCS inhibited germination of heat-activated spores in L-cysteine; the other three had no effect. GHA and βAHA both stimulated germination induced by inosine or adenosine, most probably because of slight decomposition during incubation (see below) to form glycine or β-alanine which are synergistic with the riboside germinants.

Table 1. *Effect of alanine analogues on germination of Bacillus cereus T spores initiated by various germinants*

Germination was measured by decrease in extinction of suspension at 37° as described in Methods. OCDS was used at 6 mM and the other analogues at 10 mM. None of the analogues was germinative alone.

Spores heat-activated or unheated	Germinant (concentration)	Decrease in extinction (%) during incubation for 30 min. in the presence of: No analogue (control)	OCDS	DCS	GHA	βAHA
Unheated	Inosine (1 mM)	22	4	6·5	65	56
Heat-activated	Inosine (40 μM)	33	—	—	74	55
Heat-activated	Inosine (200 μM)	69	37	69*	—	—
Heat-activated	Adenosine (1 mM)	15	—	—	31	24
Unheated	L-Cysteine (10 mM)	9	8	4	4	6
Heat-activated	L-Cysteine (10 mM)	27	30	9	23	23
Unheated	CaDPA (40 mM)†	4	2	—	—	—
Heat-activated	CaDPA (40 mM)	43	40	—	—	—
Unheated	*n*-Dodecylamine (1/5 satd.)†	42	44	40	—	—
Heat-activated	*n*-Dodecylamine (1/5 satd.)	43	35	—	—	—

* D-Cycloserine consistently caused a slight increase in the initial rate of germination of heat-activated spores in inosine, but this increase was lost after 30 min. incubation.

† Germination initiated by calcium dipicolinate (CaDPA) and *n*-dodecylamine was additionally unaffected by D-alanine (10 mM) and by D-alanine (10 mM) + OCDS (6 mM).

Effect of OCDS on germination initiated by combinations of germinants

Although L-alanine initiates germination of *Bacillus cereus* T spores and D-alanine inhibits L-alanine-initiated germination, both the D- and L-isomer will potentiate germination initiated by a germinative riboside like adenosine (Lawrence, 1955). Furthermore, other amino acids, which are not germinative alone, will potentiate the germination caused by a riboside. It was found that OCDS strikingly inhibited the potentiation of adenosine-initiated germination caused by D-alanine whilst hardly affecting potentiation caused by L-alanine (Fig. 4, 5). Similar experiments with α-aminobutyric acid, phenylalanine, serine, glycine (Table 2) and other amino acids revealed that the strong inhibition by OCDS of potentiation by D- but not by L-isomers was typical of alanine but not of other amino acids. Nevertheless, OCDS stimulated germination initiated by adenosine + some of the L-isomers of amino acids other than alanine (Table 2).

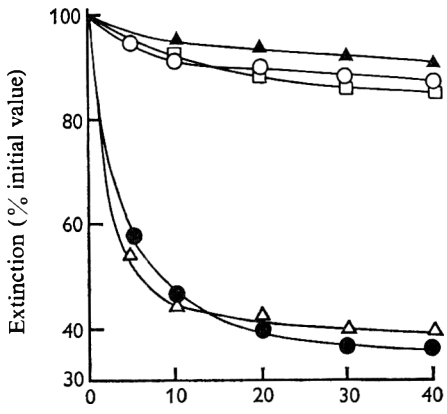

Fig. 4

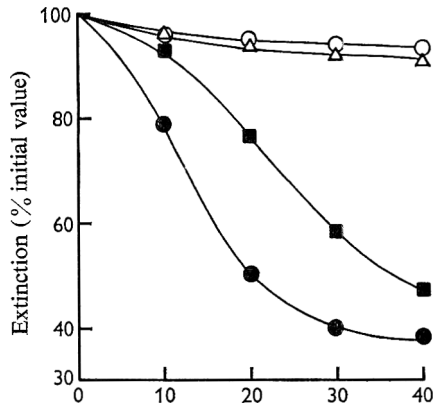

Fig. 5

Fig. 4. Effect of *O*-carbamyl-D-serine (OCDS) on L-alanine-adenosine-initiated germination of *Bacillus cereus* T spores. Heat-activated spores were germinated (see Methods) by incubation at 37° in sodium phosphate buffer (80 mM, pH 8·0) containing L-alanine (100 μM) and adenosine (100 μM) as follows: O, L-alanine alone; ▲, adenosine alone; ●, L-alanine + adenosine; □, adenosine + OCDS (2 mM); △, L-alanine + adenosine + OCDS (2 mM).

Fig. 5. Effect of OCDS on D-alanine + adenosine-initiated germination of *Bacillus cereus* T spores. Conditions as for Fig. 4 except that suspensions contained D-alanine (100 μM) and adenosine (100 μM) as follows; ○, D-alanine alone; ●, D-alanine + adenosine; △, D-alanine + adenosine + OCDS (2 mM); ■, D-alanine + adenosine + OCDS (100 μM).

Table 2. *Effect of O-carbamyl-D-serine on germination of Bacillus cereus T spores initiated by adenosine plus L- and D-amino acids*

Germination was measured as described in Table 1. The germinant mixture for unheated spores was adenosine (1 mM) + amino acid (10 mM), and for heat-activated spores was adenosine (100 μM) + amino acid (100 μM).

	Decrease in extinction (%) during incubation for 50 min. in the presence of adenosine + amino acid			
	Unheated spores		Heat-activated spores	
Amino acid	OCDS absent	OCDS present	OCDS absent	OCDS present
No addition (control)	6	7	10	20
L-Alanine	65	65	63	62
D-Alanine	50	13	63	9
L-α-Amino butyric acid	61	61	60	62
D-α-Amino butyric acid	4	10	55	55
L-Phenylalanine	61	61	35	57
D-Phenylalanine	1	3	1	3
L-Serine	30	47	35	50
D-Serine	7	7	12	26
Glycine	20	32	45	53

Stimulation of germination by hydroxylamine

Stimulation of inosine-initiated germination by GHA and βAHA (Table 1) might have resulted from partial breakdown of these analogues to form free amino acids because GHA and βAHA are known to be less stable than the other analogues in solution (Gale & Hynes, 1966). To test for breakdown, the analogues were pre-incubated in solution to allow any decomposition to occur before addition of spores. Also, the potential breakdown products alone were tested as germination stimulators.

Table 3. *Inhibition of Bacillus cereus T spore alanine racemase by alanine analogues and hydroxylamine*

The racemase assay is described in Methods. Results are expressed as % of the activity of the racemase acting on 100 mM L-alanine in the absence of the analogues.

Analogue	Concentration (mM)	Racemase activity (% of activity in analogue-free control)
O-Carbamyl-D-serine	100	7·7
	10	16·9
	0·5	79·8
D-Cycloserine	100	35·4
β-Alanyl hydroxamic acid	200	72·3
	100	86·2
Glycyl hydroxamic acid	200	81·6
	100	89·2
Hydroxylamine	10	28·8
	1	55·5
	0·1	92·5

Table 4. *Stimulation of germination of Bacillus cereus T spores by hydrolysis products of β-alanyl hydroxamic acid and glycyl hydroxamic acid*

Germination of the unheated spores was measured by fall in extinction of suspensions incubated at 30° with L-alanine (10 mM) as described in Methods.

Hydroxylamine concentration (mM)	Decrease in extinction (%) during incubation for 90 min. in the presence of hydrolysis products		
	β-Alanine- and glycine-free control	β-Alanine (10 mM)	Glycine (10 mM)
0	22	22	19
10	57	60	50
5	62	—	—
1	58	—	—
0·1	29	—	—

Pre-incubation did not increase the activity of GHA and βAHA as germination stimulants, arguing against the importance of decomposition; however, the observation was made that hydroxylamine, which would be one of the hydrolysis products of the analogues, was a powerful stimulator of L-alanine-initiated germination itself (Table 4).

Inhibition of alanine racemase by the analogues

The analogues were tested for ability to inhibit the racemization of alanine catalysed by spores of *Bacillus cereus* T. Table 3 shows that OCDS was by far the most effective racemase inhibitor, in keeping with its strong activity as a potentiator of L-alanine-initiated germination; next most effective was D-cycloserine, and least effective the hydroxamic acids. Hydroxylamine, in keeping with its stimulatory effect on L-alanine-initiated germination, was a powerful racemase inhibitor.

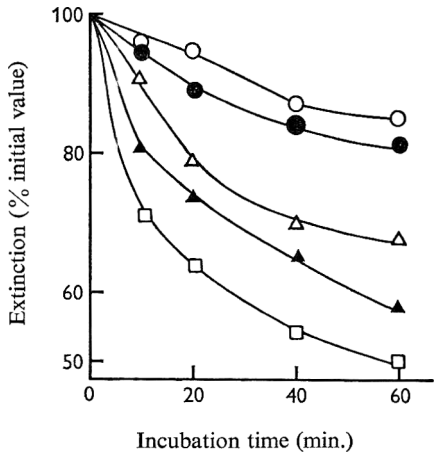

Fig. 6

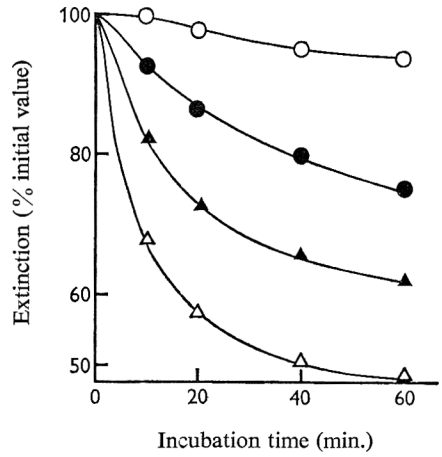

Fig. 7

Fig. 6. Effect of pre-incubating spores of *Bacillus cereus* T in the presence of glycyl hydroxamic acid. All tubes contained glycyl hydroxamic acid (10 mM) and unheated spores in sodium phosphate buffer (80 mM, pH 8·0) and were pre-incubated for various times before L-alanine (10 mM) was added to initiate germination. Pre-incubation times were: ○, no pre-incubation (spores added with alanine at zero time); ●, 1 min.; △, 5 min.; ▲, 30 min.; □, 2 hr. Qualitatively similar results were obtained with β-alanyl hydroxamic acid in place of glycyl hydroxamic acid.

Fig. 7. Binding of β-alanyl hydroxamic acid to spores of *Bacillus cereus* T. Unheated spores were preincubated with or without β-alanyl hydroxamic acid as described in Fig. 6, then either germinated immediately or washed three times by centrifugation before adding L-alanine (10 mM) to initiate germination: ○, β-alanyl hydroxamic acid-free control; ●, no pre-incubation (spores added with alanine at zero time); △, pre-incubation for 120 min.; ▲, pre-incubation for 120 min. followed by three washes. Qualitatively similar results were obtained with glycyl hydroxamic acid in place of β-alanyl hydroxamic acid.

Binding of β-alanyl hydroxamic acid and glycyl hydroxamic acid by spores

The weak inhibition of spore alanine racemase by βAHA and GHA (Table 3) was surprising in view of the stimulatory effect of these analogues on L-alanine-initiated germination (Fig. 2 and 3). Evidently the stimulation was not due to hydroxylamine formed by hydrolysis of the analogues, since pre-incubation of the analogues alone did not increase their effectiveness. However, pre-incubation of the spores in the presence of the analogues before addition of L-alanine to initiate germination did result in an increased germination rate (Fig. 6). Furthermore, when spores were pre-incubated with βAHA or GHA and then washed to remove exogenous analogue, they still germinated faster in L-alanine than did untreated spores (Fig. 7). These results strongly

suggested that βAHA and GHA, or a product formed from them, could bind to some site in spores during incubation (possibly to molecules of alanine racemase) and thereby be more effective than the simple racemase assay (Table 3) would suggest. Pre-incubation of spores with OCDS and DCS also increased their stimulation of L-alanine-initiated germination, but the increase was small compared with that observed with βAHA and GHA.

Effect of O-carbamyl-D-serine on germination of spores of different Bacillus species

Spores of eight strains representing five Bacillus species were incubated in L-alanine (10 mM) with or without OCDS (1 mM). It was found that the stimulatory effect of OCDS on germination differed very much with different spores. The *Bacillus cereus* spores tested responded most readily to the analogue (Table 5). Germination of spores of a number of the organisms shown in Table 5 was stimulated more strongly when thicker suspensions were used (equiv. about 1 mg. dry wt/ml.), suggesting that stimulation of L-alanine-initiated germination by OCDS was a general phenomenon, although much less marked with some spores than with others.

Table 5. *Stimulation of L-alanine-initiated germination of spores of various Bacillus species by O-carbamyl-D-serine*

Spores (equiv. about 40 μg. dry wt/ml.) were incubated at 37° in sodium phosphate buffer (80 mM); pH 8·0) containing L-alanine (10 mM) and with or without OCDS (1 mM). Germination was measured by decrease in extinction of suspensions as described in Methods.

	Decrease in extinction (%) during incubation for 30 min.			
	Unheated spores		Heat-activated spores	
Organism	OCDS absent	OCDS present	OCDS absent	OCDS present
B. cereus T	14	44	43	65
B. cereus PX	10	47	29	61
B. cereus NCTC 945	8	39	40	67
B. megaterium	6	12	11	23
B. subtilis A	—	—	21	36
B. subtilis (*globigii*)	29	34	44	47
B. pumilus S 3	37	47	57	65
B. polymyxa M 1	41	45	46	52

Table 6. *Growth-inhibitory activity of the analogues*

The analogues were incorporated at 25 mM and at halving dilutions down to 0·195 mM in nutrient broth inoculated with the organisms indicated. End-points (no turbidity) were read after incubation for 24 hr at 37° (see Methods).

	Minimum inhibitory concentration (mM) of analogue				
Organism	OCDS	GHA	βAHA	DCS	DAHA
Escherichia coli	6·25	12·5	25	0·39	25
Pseudomonas aeruginosa	> 25	25	25	3·1	12·5
Staphylococcus aureus	25	12·5	12·5	0·78	25
Streptococcus faecalis	> 25	> 25	> 25	3·1	> 25
Bacillus cereus T	25	25	25	0·39	25

Growth inhibitory activity of the analogues

The analogues stimulated L-alanine-initiated germination of spores in order of decreasing activity: OCDS, GHA, DCS and βAHA; D-α-alanyl hydroxamic acid (DAHA) was inactive. It was of interest to compare this order with the growth-inhibitory activities of the analogues, since they were originally developed as potential antibiotics. Table 6 shows that the two activities appear superficially to be unrelated. For example, DCS was by far the most effective inhibitor of growth (Table 6), yet not the most effective stimulator of germination; in contrast, OCDS was the most effective stimulator of germination but was relatively ineffective as an inhibitor of growth.

DISCUSSION

The analogues of alanine studied were originally isolated as antibiotics or synthesized as potential inhibitors of microbial growth. It was therefore surprising to find that they stimulated rather than inhibited germination of bacterial spores. However, the process of spore germination is peculiar in that it is essentially a degradative reaction and involves none of the complex syntheses of growing organisms; it is consequently likely that biochemical sites for inhibition of spore germination by antimicrobial agents are few. Three factors suggest that the analogues studied stimulated spore germination principally by inhibiting spore alanine racemase. First, D-alanine is known to be a powerful competitive inhibitor of germination initiated by L-alanine (Hills, 1949). Secondly, spores of some species have been shown to contain an alanine racemase which is often measurably active in the otherwise dormant, i.e. ungerminated, spore (Stewart & Halvorson, 1953), and thick suspensions of spores have been shown to inhibit their own germination in L-alanine by rapidly catalysing production of the inhibitory D-isomer (Fey *et al.* 1964). Thirdly, both DCS (Strominger, Ito & Threnn, 1960) and OCDS (Lynch & Neuhaus, 1966) were shown to inhibit alanine racemase in *Staphylococcus aureus* and *Streptococcus faecalis* respectively. DCS and OCDS also inhibited alanine racemase in *Bacillus cereus* spores (Table 3), and therefore most probably potentiated germination by this action. Further evidence was given by Krask (1961), who showed that D-cysteine (which may be regarded as another 'analogue' of D-alanine) potentiated L-alanine-initiated germination of *B. cereus* T spores and also inhibited spore alanine racemase. Stimulation of germination of thick spore suspensions by copper may also have resulted from inhibition of alanine racemase (Powell, 1957).

The two analogues βAHA and GHA are antimycobacterial agents that may act in a similar manner to DCS and OCDS in inhibiting microbial growth (Gale & Hawkins, 1965; Gale & Hynes, 1966). However, they were much less effective inhibitors of spore alanine racemase than DCS or OCDS. The surprisingly powerful stimulation of spore germination by βAHA and GHA seemed to result from binding of these analogues during incubation with the spores. Additional stimulation by formation of hydroxylamine from the analogues in the presence of spores cannot be completely ruled out, for hydroxylamine was shown by Roze & Strominger (1966) to inhibit alanine racemase in *Staphylococcus aureus* more effectively than even D-cycloserine, and was also an effective inhibitor of alanine racemase and stimulator of germination in spores of *Bacillus cereus* T (B. J. Krask, personal communication; and Table 3). D-α-Alanyl hydroxamic acid (DAHA) was tested because it appeared to be a better

structural analogue of D-alanine than either βAHA or GHA (Fig. 1), and yet its activity both as an antimicrobial agent and as a potentiator of L-alanine-initiated germination was unaccountably negligible.

The effects of alanine racemase inhibitors on germination initiated by single germinants other than L-alanine (e.g. ribosides, L-cysteine or other amino acids) were in keeping with non-involvement of the racemase, and also therefore presumably of endogenously formed L-alanine, in the metabolic pathways involved. For instance, the results argue against inosine as initiating germination by somehow triggering release of L-alanine within the spore. Krask & Fulk (1966) showed that germination of spores of *Bacillus cereus* T by L-cysteine did not involve formation of L-alanine from the cysteine, but more likely resulted from action of L-alanine dehydrogenase directly on the L-cysteine molecules; the spore enzyme is not completely specific for L-alanine (O'Connor & Halvorson, 1961*a*) and germination can be initiated by the other substrates of the enzyme (O'Connor & Halvorson, 1961*b*; Hermier & Rousseau, 1967). In such a situation one would not expect inhibition of alanine racemase to lead to stimulation of germination.

Although OCDS stimulated the germination initiated by L-alanine and had little effect on germination initiated by adenosine+L-alanine, it strongly inhibited the germination initiated by adenosine+D-alanine. This result suggested that the role of D-alanine was solely to supply L-alanine and that the L-isomer was the true synergist with adenosine; when the racemase was inhibited by OCDS no L-isomer was formed and germination was therefore arrested. OCDS did not effect germination initiated by adenosine+amino acids other than alanine in this clear cut manner. In general, OCDS slightly stimulated such germination, as it did the slow germination caused by adenosine alone (Table 2); OCDS certainly did not inhibit the activity of the D-isomers, thus suggesting that D-alanine was not an intermediate in their utilization. Lack of effect of the analogues on germination initiated by *n*-dodecylamine or calcium dipicolinate supports the concept that these reagents are not metabolized, but initiate germination by causing physico-chemical changes in spores (Rode & Foster, 1961; Riemann & Ordal, 1961).

It was interesting to find that high antibacterial activity of an analogue did not necessarily accompany high activity as a potentiator of L-alanine-initiated germination. The reason for this is probably that, whereas potentiation of germination depends principally (if not entirely) on the antiracemase activity of the analogues, the antibacterial activity depends additionally on inhibition of other enzymes which are normally involved in murein synthesis in growing organisms. For example, DCS powerfully inhibited not only alanine racemase but also D-alanyl-D-alanine synthetase (Strominger *et al.* 1960); inhibition of these two sequential enzymic steps in murein synthesis conferred high antibacterial activity on DCS. In contrast, OCDS inhibited spore alanine racemase more effectively than did DCS and was consequently a better potentiator of L-alanine-initiated germination; yet OCDS did not inhibit D-alanyl-D-alanine synthetase (Lynch & Neuhaus, 1966) and was therefore relatively less active than DCS as an antibacterial agent. Perhaps the free carboxyl group on OCDS but not on DCS contributed to antiracemase activity since OCDS is structurally closer to the free D-alanine substrate of the racemase than is DCS or the other analogues (Fig. 1). In contrast, DCS contains no free carboxyl group but a —NH—CO— link analogous to the peptide bond region of D-alanyl-D-alanine and is therefore a better

analogue of the dipeptide whose synthesis it inhibits than is OCDS. The relationships of structure of these analogues to their antibiotic activity was reviewed by Neuhaus (1967). It may be that more effective potentiators of germination could be discovered by extension of these studies with spores, but it is clear that there is no reason to assume that such potentiators would also necessarily be more effective as antibacterial agents.

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Areas of Adhesion between Wall and Membrane of *Escherichia coli*

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SUMMARY

The envelope of *Escherichia coli* B exhibited areas at which cell wall and protoplasmic membrane were intimately associated. These areas became visible in ultrathin sections after the bacteria had been fixed and embedded in plasmolysed state. At numerous areas the protoplasmic membrane was observed to adhere to the wall, while the protoplast had shrunk. Duct-like extensions of the protoplasmic membrane were thus formed. Two hundred to 400 of these wall membrane associations are found per bacterium of *E. coli* B. In a number of cells the chromosomal material is seen in close proximity or connected to a wall membrane association.

INTRODUCTION

The bacterial surface exhibits an array of very different functions: it is responsible for the priming of wall synthesis, it plays an essential part in the execution of cell division, it provides for most of the cell's mechanical stability, as well as for pathways for metabolic products and excretion of toxins, and for exhibiting specific antigens and receptor sites to which bacteriophages adsorb. To understand these various functions a structural definition of the cell surface becomes necessary. It had been shown by Weibull (1958) and McQuillen (1960) that the envelope of Gram-positive bacteria consisted of a rather amorphous outer structure which is generally referred to as 'wall' and an underlying plasma membrane. The analogue building principle, i.e. a wall and a membrane underneath, has been found in Gram-negative bacteria; however, in this group an apparent multilayering of the wall components had been demonstrated by Kellenberger & Ryter (1958) and others. The layered nature of the outer portion of the wall of *Escherichia coli* had been established using chemical and morphological techniques by Weidel, Frank & Martin (1960), and for other Gram-negative bacteria by Murray (1962). Underneath these layered components a rigid wall layer of a glycosaminopeptide had been found, together with a proteinaceous particulate substance (Martin & Frank, 1962). The glycosaminopeptide is largely responsible for the rigidity of the cell wall and also for priming of mucopolymer synthesis, whereas the outer layers of the wall containing lipoprotein and lipopolysaccharide exhibit the antigenic and endotoxic activities (Weidel & Primosigh, 1958; Salton, 1960). The study of these wall lipopolysaccharides has led to the immunological classification of bacterial strains (Westphal, Kauffman, Lüderitz & Stierlin, 1960). In addition to the presence of antigenic sites, one has also to postulate that the bacteriophage receptors have to be localized on the wall exposed to the cell's environment.

The presence of a separate membrane with selective permeability underneath the rigid wall had been demonstrated with the light microscope as early as 1903 by Fischer, who described the phenomenon of plasmolysis which consists of a shrinkage of the protoplast due to loss of water inside the cage-like wall. Plasmolysis and prevention of bursting of the protoplast by sucrose has been applied in numerous studies on membrane permeability, measurement of protoplasmic turgor and of incorporation of metabolites (see Britten, 1965), as well as in investigations on cell division (Cota-Robles, 1963) and spheroplast formation (Weibull, 1953). These investigations seemed to indicate that wall and membrane can be more or less completely separated, functionally and structurally (Salton, 1967).

We have found that in plasmolysed *E. coli* the separation of wall and membrane is incomplete. There exist numerous sites of the cell envelope at which the protoplasmic membrane stays intimately associated with the wall. The fine structure of these wall-membrane associations is described in the present report.

METHODS

Escherichia coli strain B was grown logarithmically in 10 ml. nutrient broth (1 % Bacto-tryptone, 0·5 % yeast extract, 0·5 % NaCl, 0·1 % glucose, adjusted to pH 7 with N-NaOH) at 37° with aeration (about 200 ml. air/min.), with a generation time of 25 to 30 min. until counts between 5×10^7/ml. and 2×10^8/ml. were reached. The bacteria were then plasmolysed by adding equal volumes of nutrient medium (pH 6·8 to 7·0) containing 20 or 40 % (w/v) sucrose and kept for about 2 min. at 37° with aeration. The bacteria plasmolysed almost immediately in the hypertonic environment (Bayer, 1967*b*).

The number of organisms per colony grown on nutrient agar was determined by punching-out a colony and suspending the agar piece with the bacteria in nutrient broth. The suspension was then plated on nutrient agar for colony counts. A Klett–Summerson photometer was used for turbidimetric measurements. Besides colony counts bacteria were also counted in a Neubauer chamber with a Zeiss phase-contrast microscope. For electron-microscopic studies the plasmolysed bacteria were fixed by mixing the suspension with an equal volume of the following fixative: 1 part 32 % (w/v) formaldehyde solution made out of paraformaldehyde dissolved in water + 1 part nutrient medium containing 20 % or 40 % sucrose; shortly before fixation the solution was adjusted to pH7 by adding N-NaOH. Thus the final fixing solutions contain 10 or 20 % (w/v) sucrose. Fixation was done at a temperature between 18° and 20° in centrifuge tubes. After 2 hr the bacteria were centrifuged at 7000 *g* for 15 min. in the angle head of a Sorvall centrifuge. The pellet was gently resuspended in 1 % (w/v) osmium tetraoxide solution to which sucrose, to a final concentration of 10 % or 20 %, had been added shortly before fixation, and adjusted to pH 7 with N-NaOH or with Michaelis buffer. The OsO_4 fixation lasted for 1 hr at a temperature of 18° to 20°. The suspensions were then centrifuged at 4000 *g* for 10 min. and the pellet was resuspended in an aqueous solution containing: 0·5 % OsO_4 + 0·5 % freshly prepared uranyl acetate and 10 % or 20 % sucrose. The preparations were left in this fixative overnight at a temperature of 18° to 20°. After stepwise dehydration in acetone the material was collected in pellets and embedded in Vestopal W (Jaeger, Zurich, Switzerland); grey to silver-grey sections were cut with a diamond knife in a Porter–Blum ultramicrotome equipped with an additional thermal advance or in an

LKB ultramicrotome. The sections were stained in saturated uranyl acetate solutions for 1 to 2 min. and stained further with lead hydroxide stain (Reynolds, 1963) for 1 to 2 min. in a CO_2-free atmosphere. The sections were studied and micrographs were taken on Ilford Special Lantern Contrasty plates, usually at a magnification of 40,000 in a Siemens Elmiskop I, equipped with double condenser and pointed filament. The instrument's magnification was calibrated with a cross-grating of 2160 lines/mm.

RESULTS

Viability of the bacteria in sucrose

When *Escherichia coli* B was assayed on 10% or 20% sucrose plates the same number of colonies developed after 12 hr as on control plates without sucrose. However, the size of the colonies decreased in 10% sucrose to about one half of the size of the control colonies, whereas in 20% sucrose the colonies were frequently as small as pinpoints and increased in size after 24 to 48 hr of incubation. After 2–3 days of incubation, all the colonies on the plates containing 20% sucrose had reached the size of the control colonies which had been grown for 12 hr without sucrose. The number of bacteria per colony of about 3 mm. diameter on nutrient agar plates with or without sucrose was about 5 to 6×10^8, which indicates that there was the same number of bacteria to be expected per equally sized colony. The effect of sucrose on the colony size can be attributed mainly to a decrease in the speed of growth. In liquid cultures aerated in test-tubes the results support this assumption. The generation time in nutrient broth at 37° was about 30 min. In 10% sucrose this time was only slightly increased to about 35 min., whereas in 20% sucrose the generation time rose to 68 min, For estimation of the number of bacteria by turbimetry, a calibration curve for each of the sucrose concentrations had to be made since the turbidities of the bacterial suspensions decrease with increasing concentrations of sucrose. For example: a turbimetric value corresponding to 10^8 bacteria/ml. nutrient medium corresponded to 2×10^8 bacteria/ml. nutrient medium containing 10% sucrose and to 4×10^8/ml. nutrient medium containing 20% sucrose. The concentration of bacteria was also determined by colony counts and by microscopic counts. The dimensions (length and width) of the bacteria growing in sucrose were smaller, but also rather long forms—up to 30 μ—became visible in the microscope, especially after growth in 20% sucrose.

Light microscopy

When the logarithmically growing bacteria were suddenly exposed to sucrose in concentrations of 10% or 20%, one observed in the phase-contrast microscope that in most of the bacteria the protoplast shrank almost immediately and that these forms stayed plasmolysed for about 8 min. At the end of this period the protoplasts began to swell until after 10 min. in 10% sucrose most of the forms had filled the entire visible space inside the rigid cell wall. In 20% sucrose the organisms required considerably more time for re-establishment of their former protoplasmic volume. In addition, a greater variation in the speed of recovery between individual organisms of a culture became obvious; some organisms recovered morphologically after only 8 to 10 min. whereas others needed 15 min. or longer. A few (around 2%) organisms did not seem to plasmolyse at all.

Electron microscopy

In ultrathin sections of bacteria which had been plasmolysed in sucrose the staining with uranyl acetate and lead acetate was extraordinarily difficult. Precipitates frequently developed over the area of some of the bacteria but also the background which contained no bacteria seemed to be stained and often showed a considerable increase in granularity (e.g. Pl. 1, fig. 1). Variation of staining time and concentration of the staining solution did not significantly change this tendency for precipitation.

Escherichia coli, after embedding and thin sectioning, exhibits clearly the structural elements of the wall and the protoplast typical of a Gram-negative organism. The wall consists of a pair of contrasty layers which are separated by a less dense layer; sometimes an extra dense layer becomes visible as a separate line inside the inner dense layers (Pl. 1. fig. 1); this profile has been described in recent years by a number of investigators (Kellenberger & Ryter, 1958; Glauert, 1962; Murray, 1968; DePetris, 1967).

The appearance of the wall in cross-sections seems unaltered when the bacteria were exposed to hypertonic sucrose solutions (Pl. 1, fig. 2; Pl. 2, fig. 3). The inner contour of the wall, especially its granular–globular portion, became more distinct since the protoplasmic membrane had retracted from the wall. The thickness of such a portion of the wall measured about 160 Å. In those areas where for a short distance no globular layer was detectable, the thickness of the structure was reduced to about 95 Å.

Brief exposure of the bacterium to hypertonic medium causes the protoplast to become dehydrated and shrink. A separation of the protoplast from the rigid wall's inner layer then became visible (Pl. 1, fig. 1, 2). However, in about 2 % of the bacteria a separation of wall and membrane was observed in the light microscope, even in 20 % sucrose solutions; a portion of such a bacterium is visible at top left (Pl. 1, fig. 1). In the plasmolysed bacteria the space between wall and shrunken protoplast appeared to be 'empty', with the exception that a number of protoplasmic extensions or 'ducts' showed up which maintained a connexion between wall and membrane. (The reason for our terminology is given in the Discussion.) At these areas of the wall the protoplasmic membrane was locally held back, while the main portion of the protoplast had shrunk; thus bridges or 'ducts' were formed which maintained a connexion between the protoplast and localized areas at the inside of the wall (Pl. 1, fig. 1, 2; Pl. 2, fig. 5).

In a plasmolysed bacterium the contour of the cell surface in an area of intimate contact between wall and membrane was sometimes straight but more frequently it was incurved (Pl. 1, fig. 1, 2; Pl. 2, fig. 4). The contour of the protoplasmic membrane might not change its direction at an area of wall membrane contact (Pl. 2, fig. 4) or, as in most cases, the membrane might be pulled out to a more or less elongated duct (Pl. 1, fig. 1, 2; Pl. 2, fig. 5) The ducted type in combination with a concave area of wall was most frequently observed: of 178 wall membrane associations counted on randomly picked bacteria, 115 (65 %) were of this type, whereas in 54 cases (30 %) the membrane formed a duct without visibly indenting the wall. At the deepest point of such an indentation, as well as at other types of wall membrane associations, the wall sometimes seemed to lack the granular layer.

The outer contour of the protoplast was represented by the triple-layered structure of the protoplasmic membrane (Pl. 1, fig. 1, 2; Pl. 4, fig. 9) about 90 Å in thickness. However, very often it was impossible to delineate the contour of the inner layer facing

the protoplasm because the electron densities of the fixed protoplasmic contents and the inner layer of the protoplasmic membrane were about equal (Pl. 1, fig. 2). Occasionally the protoplasmic membrane appeared to be granular or beaded. The contour of the protoplasmic membrane was continuous with the membrane contour of a duct. At the areas where a duct terminated at the wall's inner surface, the structural definition of the membrane was, in general, blurred and estimations of the thickness of the layers involved were difficult to obtain. This difficulty might be caused by an inevitable oblique-sectioning of the wavy contact area which is of smaller dimensions than the thickness of a section. In the few cases where

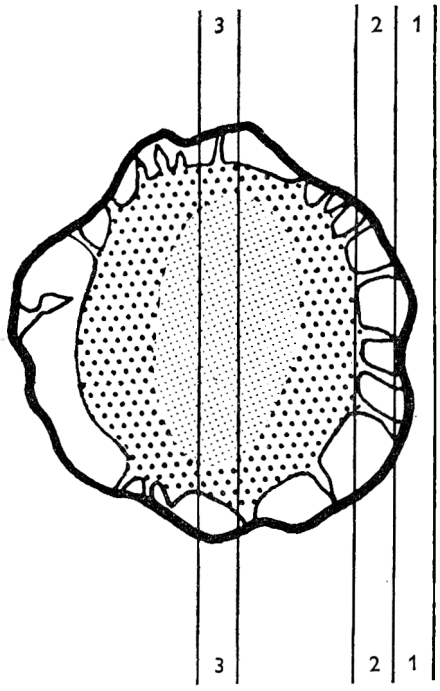

Fig 1

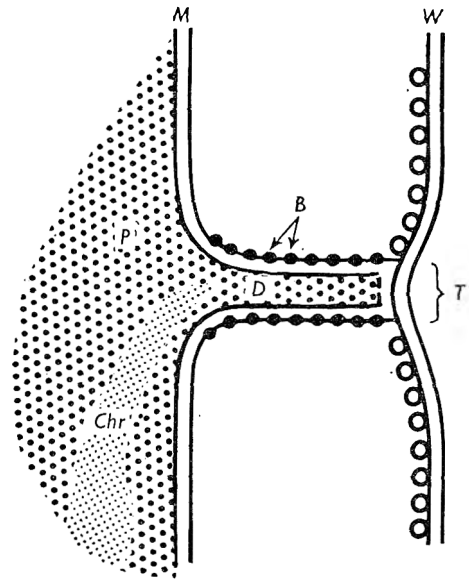

Fig. 2

Fig. 1. Diagram of various section planes through a plasmolysed cell.

Fig. 2. Schematic diagram of a wall membrane association as seen after plasmolysis. *P* = protoplast, *M* = protoplasmic membrane, *B* = 'beads' on protoplasmic membrane, *D* = protoplasmic duct, *T* = termination area of duct, *W* = wall (the circles attached to the inner 'layer' of the wall symbolize the particulate protein of the rigid layer), *Chr* = chromososome.

measurements were possible the wall showed a thickness between 95 Å (without its granular portion) and 160 Å (when the granular layer of the wall was visible). The over-all thickness of the wall membrane complex, when definable, measured 200 to 250 Å; its diameters varied from 220 Å to occasionally over 500 Å. The wall membrane complex can be defined as a zone where membrane and wall are so closely associated that even after plasmolysis no free space is apparent between the structures.

For a better orientation with respect to the section planes in question, a diagram of a cross-section through a plasmolysed cell is shown in Fig. 1, which relates roughly to Fig. 2. The assembly of the various elements of a duct in such a cross-section through

the wall, section plane 3, Fig. 1, i.e. in a section parallel to the long axis of a duct, is shown in Fig. 2 and can be observed in Pl. 1, fig. 1, 2 and Pl. 2, fig. 5.

If sections more or less tangential to the surface included the outermost surface of the wall (section plane 1, Fig. 1), the following structures were encountered: facing the environment the wall had a weakly defined contour; it seemed to fade out into the surrounding medium (Pl. 3, fig. 6*b*). There the material consisted of a fine granular and sponge-like substance. In sections along the surface one frequently observed circular areas which were scattered over the entire area of the wall (T in Pl. 3, fig. 6*b*); they are considered to represent the termination areas of the protoplasmic ducts, possibly including short portions of ducts. Their diameters measured 230 to 270 Å. In addition, there were other structures visible in these sections: occasionally wall extrusions (L in Pl. 3, fig. 6 and 7) were seen as well-defined circular or elliptical elements 150 to 200 Å in diameter (L in Pl. 3, fig. 6) which may be regarded as sections of wall lipid which is normally found on the surface of *Escherichia coli* (Bayer & Anderson, 1965; DePetris, 1967). In sections along the cell surface channel-like structures were occasionally observed in the wall (*Ch* in Pl. 3, fig. 6). They measured 85 to 90 Å across.

When the area between wall and shrunken protoplast was sectioned (section plane 2, Fig. 1) the only structures visible were cross-sections of the connecting protoplasmic ducts. In general the cross-sections of these ducts appeared to be more or less circular in shape (Pl. 3, fig. 7). The ducts measured 200 to 350 Å across, usually 240 to 280 Å, and were composed of the triple-layered membrane of a thickness about 90 Å (Pl. 2, fig. 5), which agrees with the dimensions of the protoplasmic membrane at other locations of the protoplast. The tubelike structure of a duct showed a central core of a diameter 40 Å or more (Pl. 2, fig. 5, left duct) which seemed frequently to be 'plugged' with material (Pl. 3, fig. 7, white arrows), sometimes with a dark dot in the centre (Pl. 3, fig. 7, insert). The length of a duct depended on the extent of plasmolysis and also on the local contour of the wall. The membranes in the ducts often exhibited a beaded structure, in which the 'beads' are shown in higher contrast (Pl. 3, fig. 7, insert) with a diameter of 25 to 30 Å.

Longitudinal sections and serial sections showed that the distribution of the connecting ducts over the entire bacterium seemed to be random (Pl. 1, fig. 1, 2; Pl. 3, fig. 7). At the poles of the bacteria the ducts were often longer than at other areas. If one assumes the length of the bacterium to be 1·5 to 2 μ and the section thickness to range between 600 Å for grey-coloured sections and 800 Å for silver-coloured sections (Peachy, 1958; Bachmann & Sitte, 1958), the total number of the wall–membrane connexions per bacterium can be estimated as 200 to 400.

The protoplasmic contents were condensed after plasmolysis as compared to normal growth conditions. The ribosomes were closely packed and the space between them appeared to be much denser than comparable areas of control bacteria. Thus, the contrast variation in the protoplasm was decreased (Pl. 1, figs. 1, 2; Pl. 4, fig. 8). The fibrous chromosomal apparatus became clearly visible against this dense protoplasm. Often portions or strands of 'light' material appeared to extend from the chromosome through the dense protoplasmic contents and to aim at some of the areas of wall membrane associations (Pl. 1, fig. 1; Pl. 4, fig. 8). In sets of serial sections through 12 cells of different preparations it was observed that the chromosome membrane 'contact' occurred at more than one site per cell (Pl. 4, fig. 9).

DISCUSSION

Under osmotic conditions normally present in cultures, cell wall and protoplasmic membrane of *Escherichia coli* B are found in more or less close contact; this applies also for strain C 1 (Bayer, unpublished). Presumably, the membrane is forced against the mechanically rigid wall by the turgor of the protoplast, the pressure of which has been determined by Mitchell & Moyle (1956) and by McQuillen (1960) to be 3 to 5 kg./cm.^{2}. Only occasionally does one find areas where a slight separation of protoplast and wall becomes visible and there one might be able to observe areas where wall and membrane adhere to one another. However, these areas become conspicuous after osmotic shrinkage of the protoplast. Similar structures have previously been described as protoplasmic extensions by Cota-Robles (1963) and Birdsell & Cota-Robels (1967) in connexion with investigations on cell divisions and lysis of *E. coli*. Connexions or 'bridges' between membrane and wall have also been reported for a number of other micro-organisms like streptomyces (Glauert & Hopwood, 1960), *Bacillus subtilis* (Glauert *et al.* 1961) and Listeria (Edwards & Stevens, 1963). Since the cells have not been plasmolysed, a comparison of their bridge-like structures and the 'ducts' of our report becomes difficult. In preparations of *E. coli* the wall frequently exhibits funnel-like invaginations at the sites of wall membrane attachment (Pl. 1, fig. 1; Pl. 2, fig. 5); these structures can be interpreted as being caused by the pulling forces to which the connexions are subjected while the protoplast retracts. They might correspond to the 'dimpled holes' seen in cell walls prepared by the critical-point method (Anderson & Oster, 1954).

The beaded appearance of the protoplasmic membrane in some areas could very well represent an alignment of membrane constituents similar to those described by Sjöstrand (1963). However, such an interpretation should be regarded as tentative until more evidence for the existence of the micellar arrangement of the protoplasmic membrane is available (Robertson, 1966).

The areas of wall membrane contact might be related to the centres of wall synthesis which have been shown to be about equally numerous and also randomly distributed over the entire wall of *Escherichia coli* (Bayer, 1967*a*). One might, tentatively, assume that the areas of wall membrane contact represent mechanically weak areas of the wall at which the cells can be injured by osmotic shock (Bayer, 1967*b*). It has been shown that a light osmotic shock releases a number of enzymes and nucleotides (Heppel, 1967) and amino acid binding proteins (Piperno & Oxender, 1966), suggesting that these substances are membrane-bound or located near the surface of the organism. The reports of Anraku (1966) and Fox, Carter & Kennedy (1967), on reduction of galactose transfer systems after osmotic shock, would permit the same conclusion. The intimate junction of wall and membrane at these areas would also account for the apparent difficulties in preparing 'pure' wall preparations or 'pure' membranes (Salton, 1967).

It has been demonstrated that the bacteriophages T 1 to T 7 adsorb almost exclusively to those areas of the wall which show an association between wall and membrane (Bayer, 1968). The transport of the injected nucleic acids is likely to occur through the protoplasmic 'ducts'. Because of this we prefer the term 'duct' to that of 'extension' or 'bridge'.

The presence of connexions of the protoplasmic ducts to the cell's chromosomal

apparatus is suggested by pictures like Pl. 4, fig. 8, 9. Such an arrangement would resemble observations of Jacob, Ryter & Cuzin (1966), who suggested an anchoring of the chromosome at the membrane or its derivatives in *Bacillus subtilis*. Ryter & Jacob (1966) and Ryter (1968) have also shown that the nuclear material comes in close contact with the membrane of *Escherichia coli*. Application of plasmolysis makes it easily possible to discern structurally the contact areas of chromosome and membrane from the residual portions of the cell envelope. Much more material has to be investigated before one can decide whether all the chromosomal contacts with the membrane occur at a wall–membrane association. One might derive from a number of our micrographs that there seem to exist more than one zone in *E. coli* at which the chromosomal apparatus comes in close proximity to a wall-membrane association. Since these cells are growing rapidly and therefore contain a multiple chromosome set, the suggested value of one anchoring area per cell (Ryter, 1968) might still be applicable. However, these observations have to be backed by cytochemical and biochemical work before firm conclusions can be drawn.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Plasmolysed dividing *Escherichia coli* B in an almost longitudinal section. Portion of an unplasmolysed cell at upper left portion of picture. Note the numerous associations between wall and membrane. The bar represents 1000 Å in this and the following figures. × 62,000.

Fig. 2. Cross-section of *E. coli* B after 2 min. in 20% sucrose. × 140,000.

PLATE 2

Fig. 3. Typical view of cross-section through the wall of a plasmolysed *E. coli*. The granular layer faces the protoplast which has, in this figure, retracted beyond the area shown. × 250,000.

Fig. 4. Wall contour seems to lack granular layer at zone of contact with membrane. × 280,000.

Fig. 5. Longitudinal and oblique section through 'ducts'. *P* = Protoplast; *W* = wall. × 280,000.

PLATE 3

Fig. 6. *E. coli* wall. Insert (*a*) shows oblique cut of plasmolysed *E. coli*; the area inside the 'rectangle' represents an almost tangential section which is shown enlarged to × 220,000 in (*b*). L = finger-like extrusion of the flexible surface layers; *Ch* = channel-like structures. For further symbols see legend to Fig. 2.

Fig. 7. Plasmolysed cell in tangential section with ducts crossing the space between wall and retracted protoplast. White arrows point to some of the cross-sections of ducts. *L* = finger-like extrusions of the surface layers. Insert shows a cross-section of a duct with a central 'plug'. × 220,000.

PLATE 4

Fig. 8. Chromosomal area appears to be connected to the areas where ducts originate; × 80,000.

Fig. 9. Chromosome in close contact with the cell membrane at two sites; the cell is only slightly plasmolysed. × 55,000.

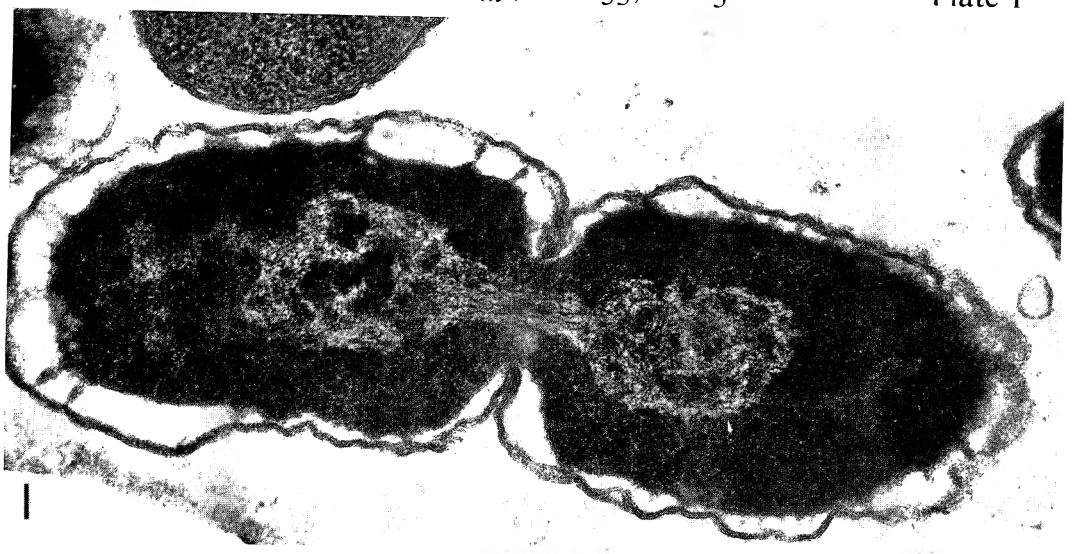


Fig. 1

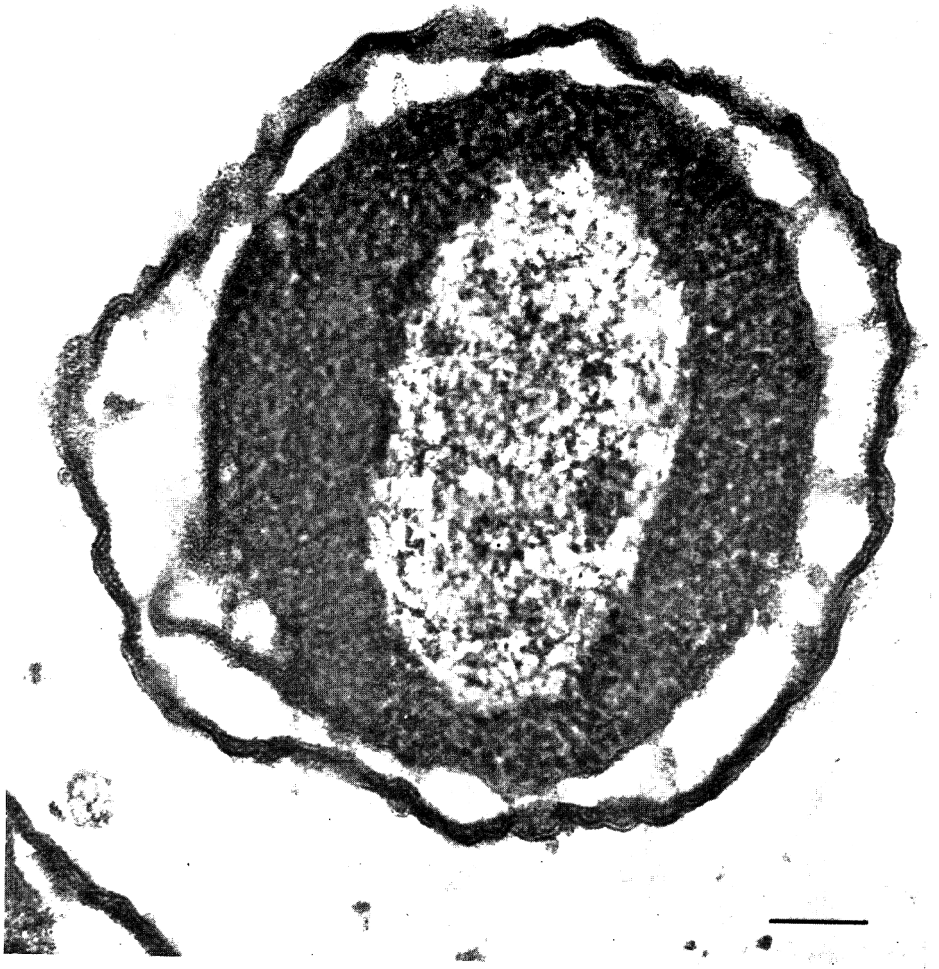


Fig. 2

(*Facing p.* 404)

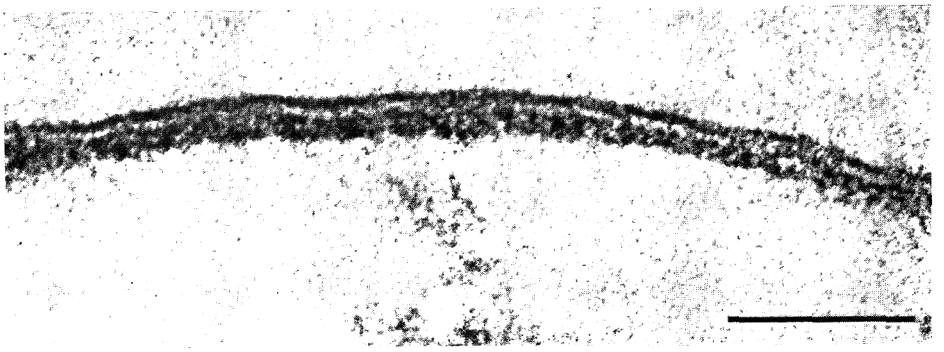


Fig. 3

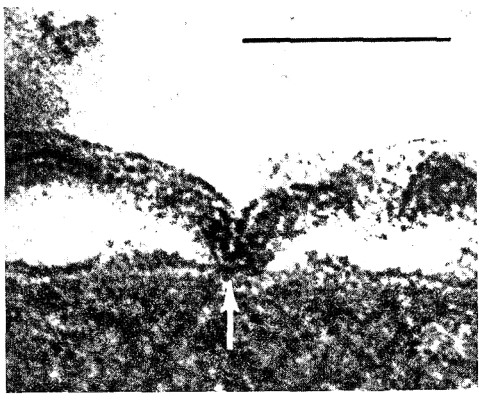
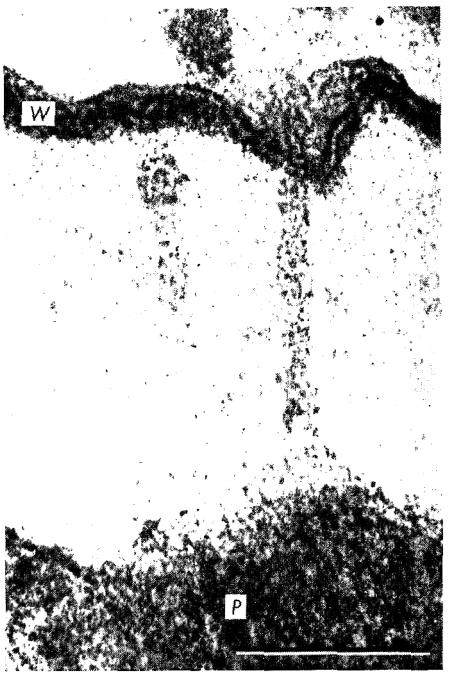


Fig. 4



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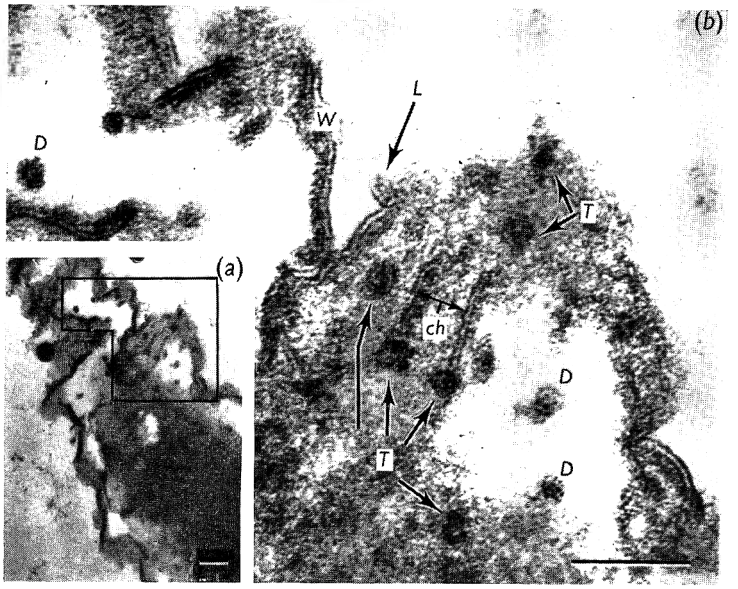

Fig. 6

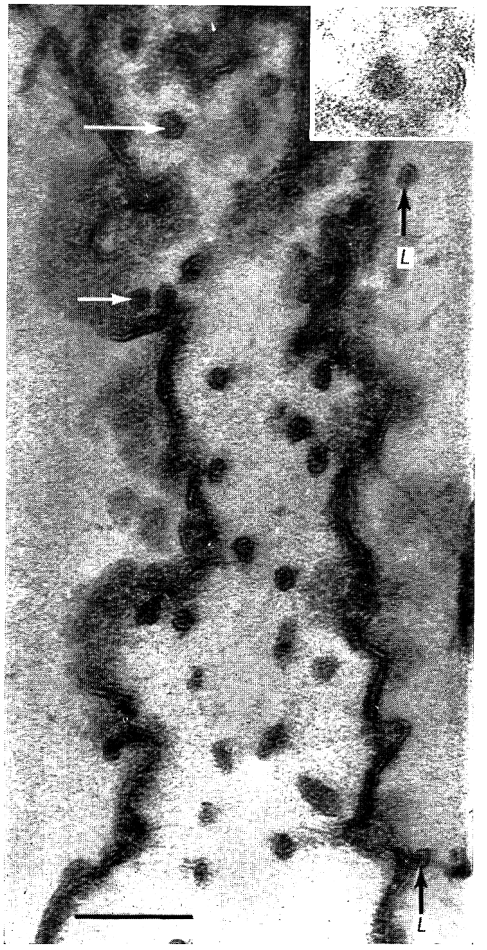

Fig. 7

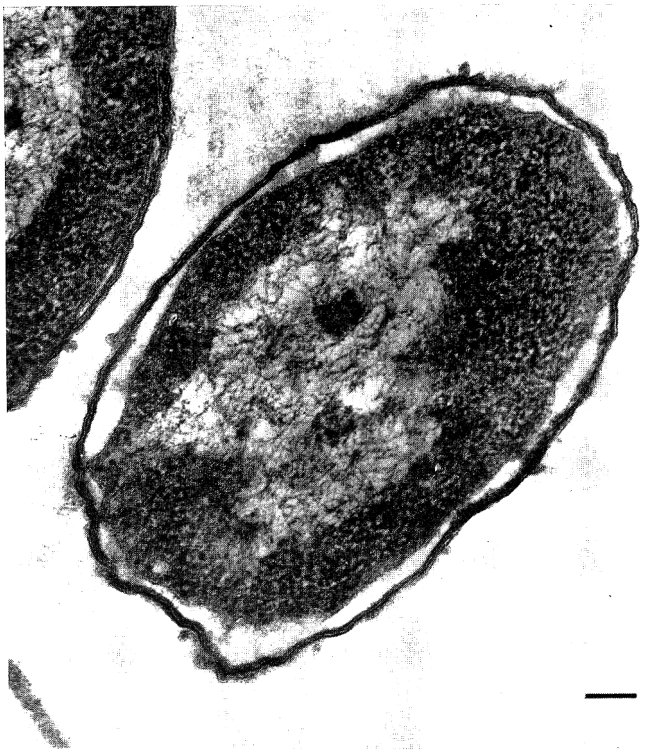

Fig. 8

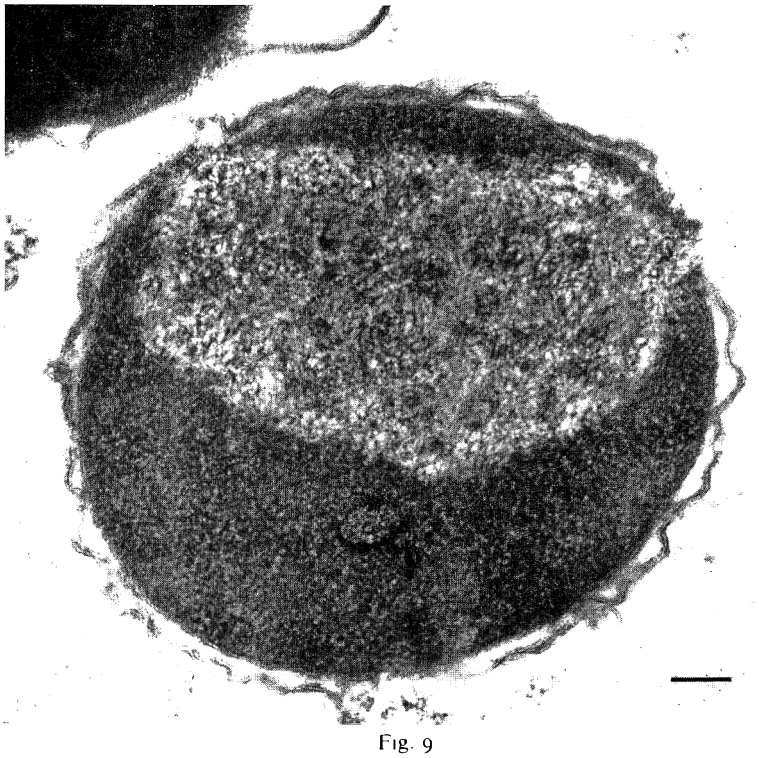

Fig. 9

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Esterases and Other Soluble Proteins of Some Lactic Acid Bacteria

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SUMMARY

Electrophoresis patterns of soluble protein of 34 strains and esterases of 113 strains of lactic acid bacteria were determined. Similar protein patterns were obtained for the three species of lactic acid streptococci; with the lactobacilli most species gave constant species-specific patterns, but *Lactobacillus acidophilus* and *L. delbrueckii* strains differed markedly among themselves. Esterase patterns of lactic streptococci were generally species specific. Among the lactobacilli the thermobacteria had weak esterase activity which was only species specific for *L. lactis*, *L. leichmanii* and *L. salivarius*; in the streptobacteria, *L. casei* had a very consistent esterase pattern, whereas *L. plantarum* had very different patterns within the species; the unclassified strains were different from each other; in the betabacteria activity was weak and no consistent pattern of bands occurred. Leuconostocs grouped in patterns corresponding to their physiological groups. Esterases of a streptococcus and a lactobacillus examined were classified as ali esterases. When ten strains of lactic acid bacteria were tested for substrate specificity, nine of them had a higher activity against α-naphthyl acetate than against the butyrate and caprylate. A rapid test for esterase activity of whole organisms is described.

INTRODUCTION

Lactic acid bacteria have been differentiated by physiological and serological tests into several well-defined groups or species, but a further understanding of their relationships might result if the electrophoretic patterns of their esterases and other soluble proteins were compared, as has already been shown for other groups of bacteria (Norris, 1964; Cann & Willox, 1965; Lund, 1965; Robinson, 1966). These lactic acid bacteria occur in large numbers in Cheddar cheese and the presence of esterases in different strains of these organisms might effect the breakdown of ester linkages of substances present in the cheese, and produce compounds contributing to the cheese flavour (Reiter, Fryer, Sharpe & Lawrence, 1966; Reiter *et al.* 1967). The relative activity of the different esterases might determine the quality of flavour. In experiments reported here, the multiple esterases of group N streptococci, lactobacilli and leuconostocs were studied, and the soluble protein electrophoretic patterns of some of the group N streptococci and the lactobacilli determined.

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METHODS

Organisms. The following 113 strains of lactic acid bacteria were used: *Streptococcus cremoris* HP, OP4, TEM2, RW, YP7, K, FH, HS2, UD5, AMI, TR, D9, R1, ML1, KH, 924, E8, ML2, R6, 803; *S. lactis* SC2, SC6, SC10, M2S1, ML3, 712; *S. diacetilactis* DRC1, DRC2; *Lactobacillus casei* C2, C4, C5, C6, C7, C9, C12, C20, C22, C24, C25, C42; *L. casei* var. *rhamnosus* C3, C10, C16, C34; *L. casei* var. *alactosus* C31; *L. plantarum* P1, P5, P12, AR3, A164; unclassified streptobacteria K44, A41, A101, D45, V4, V5; *L. helveticus* H4, H5, H17; *L. jugurti* J2, J4, J8; *L. bulgaricus* B4, B8, B17; *L. lactis* L1, L3, L19, AH7; *L. acidophilus* A1, A4, A15; *L. leichmannii* LE2, LE3, LE4; *L. delbrueckii* D2, D6, D10; *L. salivarius* SAL1, SAL5, SAL9, SAL10, SAL11, SAL13; *L. fermenti* F1, F15, F38; *L. buchneri* BC1, BC5, *L. brevis* X1, X2, X5; *L. cellobiosus* G1, G2, G3; *L. pastorianus* T1, T9; *Leuconostoc cremoris* NCDO 705, 828, 543; *Ln. lactis* NCDO 534; *Ln. dextranicum* NCDO 529, *Ln. mesenteroides* NCDO 803, 869, 870, 871, 516, 518, 523, 530, 551, 553.

All the test cultures were from a stock collection at the National Institute for Research in Dairying or from the National Collection of Dairy Organisms.

Culture procedures. Lactic streptococci were grown for 18 hr at 30° in 500 ml. MRS broth (de Man, Rogosa & Sharpe, 1960). With the lactobacilli, thermobacteria and *L. fermenti* were grown for 18 hr at 37° in 500 ml. MRS broth (de Man, Rogosa & Sharpe, 1960). The other betabacteria and the streptobacteria were grown for 18 hr at 30°. Leuconostocs were incubated for 18 hr at 30° in 500 ml. MRS broth containing 0·05 % cysteine (Dr E. I. Garvie, personal communication). Incubation periods were for 36 to 40 hr for some slow-growing strains of lactobacilli and leuconostocs (e.g. F15, X1, BC5, T1, 705).

Preparation of cell-free extracts. The bacteria were harvested by centrifugation, washed three times with chilled physiological saline or 0·1 M-phosphate buffer (pH 8·0) and suspended in 4 to 10 ml. saline. After the addition of Ballotini beads, the bacteria were disintegrated for 15 to 30 min. by Soniprobe type 1130 A (Dawe Instruments Ltd.) the containers being immersed in solid CO_2+methanol (−20°). The extracts were clarified by centrifugation and stored at −20° until required. The extracts contained 10 to 20 mg. protein/ml. estimated by the biuret micro-method (Itzhaki & Gill, 1964) with crystallized bovine plasma albumin as standard.

Electrophoresis of proteins and esterases. The cell-free extracts were analysed by electrophoresis in polyacrylamide gels. The gel preparation, electrophoresis, protein and esterase staining were done mainly according to the procedures described by Lund (1965). Samples were applied by absorbing 6 to 8 μml. of extract on pieces of Whatman 3 MM filter paper and inserting them into the gels. To detect very faint esterase bands as with *Lactobacillus casei* it was necessary to apply 30 to 50 μml. of extract. One % solution of α-naphthyl acetate, -butyrate, -caprylate or -laurate were used as substrates for detecting esterases. The solutions of the caprylate and laurate derivatives contained propylene glycol to give good stability (Gomori, 1953). Fast blue B salt was used as coupling dye to indicate hydrolysis.

Esterase stain. Since pH 8·0 is known to be optimal for the activity of some esterases, the optimum reaction of the staining solution was investigated. When the Fast Blue B salt was dissolved together with substrate in a buffer according to the procedure described by Lawrence, Melnick & Weimer (1960) the best result was obtained at the originally recommended value of pH 6·4, because the dye was rather unstable at the

higher pH value. It was possible and sometimes useful to separate the ester hydrolysis (at pH 6·4 to 8·0 for 1 hr at room temperature) from the following diazo-coupling (at pH 6·4). The apparent rate of hydrolysis by a majority of the esterases examined was practically the same in the range pH 6·4 to 8·0. Test cultures used were *Lactobacillus casei* C5, C9, *L. plantarum* P1, *L. helveticus* H17 and *Streptococcus cremoris* HP. Accordingly, in the present work, the pH value of the staining solution within the above-mentioned range was not so important, because the gel itself contained tris citrate buffer (pH 8·65). When compared with each other as coupling dye, Fast blue B salt gave better colour development than Fast blue BB or RR salts.

Comparison between polyacrylamide and starch gels. Lund (1965) pointed out that faint esterase bands seem to be more sensitively detected on starch gel than on polyacrylamide gel. When we compared the esterase patterns on both gels, using cell-free extracts of *Lactobacillus casei* C9, *L. plantarum* P5, *Streptococcus lactis* ML3, *S. diacetilactis* DRC1, *S. cremoris* 924, and *Leuconostoc cremoris* 828, the same or better results were obtained in the polyacrylamide gel. Polyacrylamide gels could not be used for quantitative work because it was almost impossible to extract the developed colour substance from the gel. Quantitative determinations of esterase activities of *L. casei* C9 were therefore made after starch-gel electrophoresis (Wright & Keck, 1961). The starch-gel slices were flooded separately with the staining solutions containing α-naphthyl acetate or butyrate. The reaction bands were eluted with *n*-amyl acetate + ethanol (1 + 1 by vol.) and determined colorimetrically at 500 mμ. Relative activities for acetate and butyrate esters were as follows: prominent esterase, 100 and 28; butyrate-specific, minor esterase, 3 and 17.

Growth media. The influence of different media upon the esterase patterns of lactobacilli were examined by using *Lactobacillus casei* C2, C9 and *L. plantarum* P1 as test organisms. Media used were as follows: APT (Evans & Niven, 1951), Rogosa (Efthymiou & Arne Hansen, 1962), MRS broth, MRS broth with glucose decreased to 0·5 % and MRS broth with Bacto peptone instead of Oxoid peptone. No significant differences were detected. Nor was any qualitative difference noted between the esterase patterns of lactic streptococci grown in glucose 1 % Lemco broth (GLB) or in GLB + 0·2 % yeast extract (Oxoid). The same pattern was obtained with *Leuconostoc mesenteroides* 869 harvested from MRSB + 0·05 % cysteine or from YG citrate broth (Garvie, 1960).

RESULTS

Soluble protein electrophoretic patterns of lactic acid bacteria

Streptococci. The variations in the patterns of soluble protein between the three species of lactic streptococci were small. Representative patterns of *Streptococcus cremoris*, *S. lactis* and *S. diacetilactis* were very similar, although a few protein bands in the *S. lactis* pattern were not detected in that of *S. cremoris*.

Lactobacilli. Most of the species gave consistent characteristic patterns which were different for each species. *Lactobacillus helveticus* and *L. jugurti*, which are closely related to each other, showed similar but not identical patterns and so did *L. lactis* and *L. bulgaricus*, two other closely related species. Six strains of *L. casei* showed identical patterns apart from two very weak bands; there was no difference between patterns of strains belonging to serological groups B and C.

L. plantarum strains showed a similar overall pattern with a few weak bands dif-

ferent. However, the protein patterns of *L. acidophilus* and *L. delbrueckii*, particularly the latter, showed considerable variation between strains: among the heterofermentative strains examined *L. cellobiosus* differed markedly from *L. buchneri*.

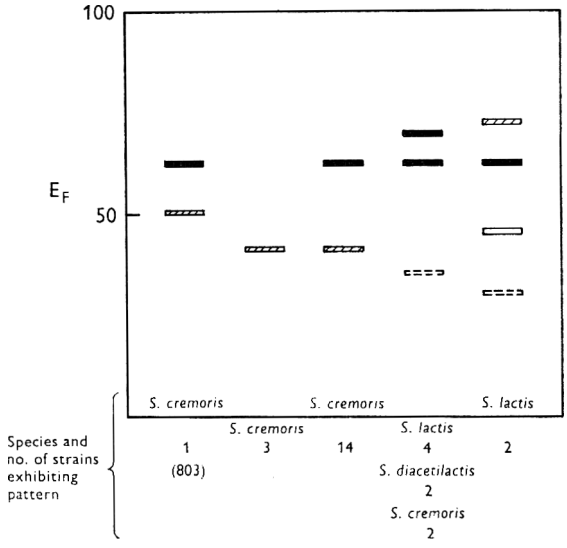

Fig. 1. Esterase patterns of lactic streptococci.

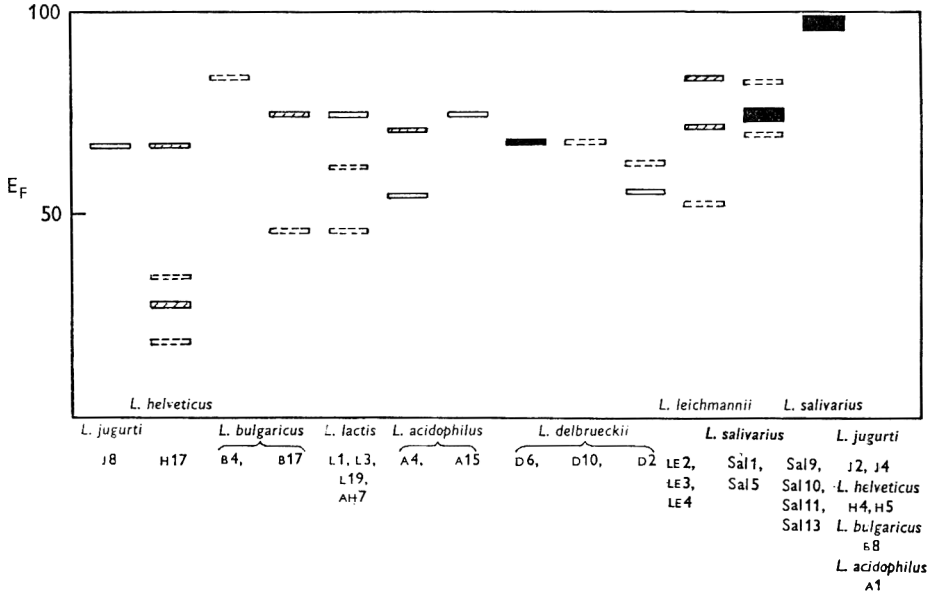

Fig. 2. Esterase patterns of thermobacteria.

Esterase electrophoretic patterns of lactic acid bacteria

Streptococci. Most of the lactic streptococci appeared to have a common esterase with a mobility of E_F 62 as shown diagrammatically in Fig. 1, only in the extracts of three strains of *Streptococcus cremoris* was this common band either not detected or

extremely weak. In general the species *S. lactis* appeared to possess a more complex esterase pattern than the species of *S. cremoris*. *Streptococcus diacetilactis*, which is physiologically nearest to *S. lactis*, had the same esterase pattern as this species. Two cultures of *S. cremoris* also had this pattern, but it was later found that these two cultures resembled *S. lactis* in ability to grow in glucose 1 % Lemco broth containing 4 % NaCl, and in fermenting maltose, but resembled *S. cremoris* in being unable to grow in milk at 37° or above. *Streptococcus cremoris* 803 also seemed to be atypical, fermenting and clotting milk at 37°.

Lactobacillus: thermobacteria. Generally speaking, thermobacteria were weak in esterase activity under our experimental conditions. For six (H4, H5, J2, J4, B8, A1) of 28 strains belonging to the eight species tested, no esterases were detected (Fig. 2). Fifteen strains possessed one to four esterases which were generally very weak in activity. *Lactobacillus delbrueckii* D6 and six strains of *L. salivarius* showed a relatively strong band of esterase.

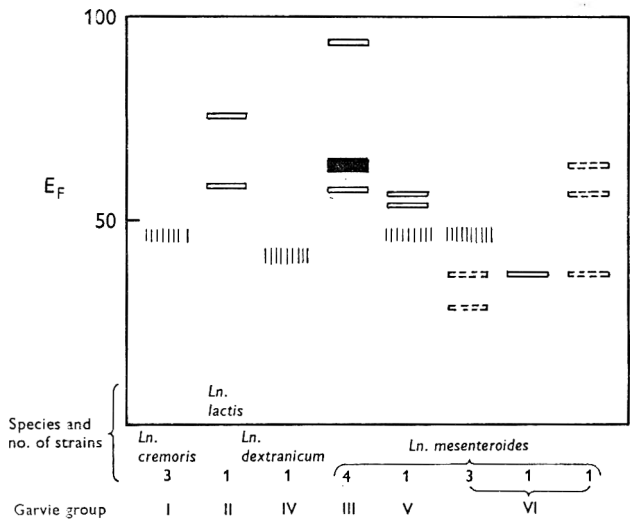

Fig. 3. Esterase patterns of leuconostocs.

All the strains of *Lactobacillus lactis* and *L. leichmannii* showed identical esterase patterns. No species-specific pattern was observed with the following six species: *L. helveticus*, *L. jugurti*, *L. bulgaricus*, *L. acidophilus*, *L. delbrueckii*, *L. salivarius*. Different incubation temperatures, i.e. 48 hr at 25° or 18 hr at 37° had no effect upon the esterase patterns of strains *L. acidophilus* A15 or *L. bulgaricus* B4.

Lactobacillus: streptobacteria. Sixteen of 17 strains of *Lactobacillus casei* showed the same esterase pattern which was characterised by a highly active band at E_F 59. No difference in the esterase patterns was observed among their three varieties, i.e. *L. casei* var. *casei*, var. *rhamnosus* and var. *alactosus*, nor between strains belonging to the different serological groups B and C (Sharpe & Wheater, 1957). Only one strain, C12, had a distinctive esterase pattern and this strain possessed atypical physiological characteristics, not fermenting mannitol or melezitose. In addition to the above-mentioned prominent band, one or two minor esterases were detected in the extracts of *L. casei*. For example, a very faint esterase band was usually observed at E_F 70.

Species-specific patterns of *L. plantarum* were not consistently obtained; this was in contrast to the observations with *L. casei*.

There are many strains of streptobacteria which grow at low temperature and are homofermentative, but are neither *Lactobacillus casei* nor *L. plantarum* (Naylor & Sharpe, 1958). Some of these unclassified strains were examined; strains A1ɔ1, K44, D45 and V4 were found to be quite different from *L. casei* in esterase pattern. A few strains (e.g. A41, V5) of this group showed no esterase activity under our conditions.

Generally speaking, the esterase activities of streptobacteria, especially *L. casei*, were higher than those of other lactobacilli with the exception of *L. salivarius* and of lactic streptococci.

Lactobacillus: betabacteria. No species-specific esterase pattern was obtained with the following organisms: *Lactobacillus fermenti*, *L. buchneri*, *L. brevis*, *L. cellobiosus*, *L. pastorianus*. The esterase activities were generally weak in these bacteria excepting *L. cellobiosus*. It is of interest that *L. cellobiosus* and *L. salivarius*, both of which were originally isolated from saliva, showed relatively high esterase activities.

Leuconostocs. All three test strains of *Leuconostoc cremoris* had an esterase at E_F 46; this was only weakly active and gave a diffuse zone (Fig. 3). Ten strains of *Ln. mesenteroides* gave two groups by the esterase analysis, four of them had a distinctive esterase pattern, the characteristic feature being a highly active esterase band at E_F 64; all these strains belong to Garvie's (1960) physiological group III. Six other strains of *Ln. mesenteroides* (Garvie's groups V and VI) were variable in esterase pattern, although the ill-defined esterase was generally detected at the same position as that for *Ln. cremoris*. One strain each of *Ln. lactis* and *Ln. dextranicum* examined gave different esterase pattern from the other leuconostocs.

Heat inactivation and inhibition of esterases

The esterases of several lactic acid bacteria (*Streptococcus cremoris* HP, *S. lactis* SC2, *Lactobacillus casei* C2, C6, C9, C10) were inactivated at 60° and 65° for 10 min. Esterases of *S. cremoris* ML3 and *L. casei* C9 were inhibited by 10^{-4} M difluorophosphate (DFP) but resistant to 10^{-4} M *c*-serine sulphate, 10^{-4} M-*o*-iodosobenzoate, 10^{-4} M-*p*-hydroxy mercuribenzoate and 10^{-3} M-EDTA). These results suggest that these esterases are ali esterases (Augustinsson, 1958, 1961).

Substrate specificities of esterases

Most of the esterases of lactobacilli which were tested had greater activity against α-naphthyl acetate, -butyrate and -caprylate in decreasing order. The esterases of some lactic streptococci hydrolysed α-naphthyl acetate and -butyrate to the same extent. The esterase of *Leuconostoc cremoris* 543 showed greater activity against the butyrate ester than against the acetate. However, one should be careful when estimating the substrate specificity by gel electrophoresis, because it is more difficult for a substrate of low solubility, such as α-naphthyl laurate, to diffuse to the enzyme in the polyacrylamide gel.

Esterase in the culture supernatant fluid of Lactobacillus casei

It was suggested by Stock, Uriel & Grabar (1961) that bacterial esterase and lipase Pollock, 1962; Lawrence, Fryer & Reiter, 1967) may be extracellular enzymes. To investigate this point *Lactobacillus casei* C7 was grownin MRS broth at 37° for 33 hr

and the esterase activities of the samples of the fluid supernatant examined at intervals, by adding ammonium sulphate to samples to 2/3 saturation and examining electrophoretically aqueous solutions of the precipitates in 0·1 M-phosphate buffer (pH 8·0). Esterase activity was not detected in the supernatant fluid of the *L. casei* culture during incubation up to 33 hr. This result suggested that esterase liberation was negligible in a *L. casei* culture. The change of pH value of the culture during growth is presented in Table 1. Such a pH change might have some influence upon the esterase present. When the cell-free extract was adjusted to pH 4·1 with HCl and incubated for 2 hr at 37° the greater part of the esterase was precipitated. This result does not mean that the isoelectric point of the esterase is pH 4·1; co-precipitation with other proteins in the cell-free extract, which contained about 20 mg. protein/ml., might be possible; also, the inactivation of enzymes liberated might not be unexpected at pH 3·8. To eliminate effects of low pH value, a culture of the same organism (*L. casei* C7) was held at pH 6·5 by adding NaOH. Esterase activity was then observed in the supernatant fluid after 17 hr of incubation. The electrophoretic mobility of the liberated esterase was the same as that of the main esterase (E_F 59) found after disrupting the bacteria. The proportion of the esterase activity which appeared in the supernatant fluid was estimated to be only 1 to 3 % of the cell-bound enzyme.

Table 1. *Detection of esterase activity in the supernatant fluid of cultures of Lactobacillus casei strain C7*

		Time of incubation (hr) at 37°			
		8	17	26	33
MRS broth, pH unadjusted	Esterase activity	–	–	–	–
	pH	5·02	4·05	3·83	3·75
MRS broth, pH adjusted	Esterase activity	±	+	+	+*
	pH	6·44	6·43	6·53	6·50

* Less than 3 % of the activity of cell-bound esterase.

Effect of trypsin on esterases of Lactobacillus casei

Trypsin destroyed the esterases in the cell-free extract of *Lactobacillus casei* C7. However, when washed whole bacteria were suspended in 0·1 M-phosphate buffer (pH 8·0) containing 2 mg. Armour crystallized trypsin/ml. and incubated at 37° for 4 hr, no change occurred in the esterase activity. Accordingly the esterase in the whole cells seems to be protected from trypsin action.

Esterase activity of whole bacteria

The intensity of the colour reaction produced by a suspension of whole bacteria on paper impregnated with α-naphthyl acetate and 0·02 % of Fast blue salt correlated approximately with the esterase activities in cell-free extracts. About 60 strains of lactobacilli, streptococci and leuconostocs were compared by these two methods. *Lactobacillus casei*, *L. salivarius* and *L. cellobiosus* rapidly produced strong pink to red colours, while the weakly esterase positive or apparently negative strains such as H5, J2, B8, F1, A41 produced little or no colour. These results were further confirmed by using a 0·02 % solution of indoxyl acetate as substrate (Barnett & Seligman, 1951; Clarke & Steel, 1966). Esterase-active organisms such as *L. casei* and *L. salivarius*

produced in 2–3 min. the deep blue colour of indican. Since control suspensions heated to 100° for 2 min. did not produce any colour, it was concluded that the reaction was caused by esterase activity.

DISCUSSION

Similarities in the electrophoretic patterns of soluble proteins such as those observed with the lactic streptococci may indicate similarity in the metabolism of the organisms, which is likely with these three species. The same applies to species of lactobacillus, where similar protein patterns were found within a species. Where there are marked differences of protein pattern within a species, as with *Lactobacillus delbrueckii*, perhaps further differentiation could be made by physiological and biochemical tests.

The esterase patterns confirm that esterase typing can help in the identification of some species of lactic acid bacteria. It seems possible to distinguish between *Streptococcus cremoris* and *S. lactis* by their characteristic esterase patterns. This is very similar to Lund's (1965) finding with *S. faecalis* and *S. faecium*. *Lactobacillus casei* strains showed a distinctive esterase pattern which may be helpful in identifying this species.

Most of the thermobacteria were weak in esterase activity under our conditions and few species-specific patterns were observed. Similarly, in the betabacteria the esterase activities were generally weak and the pattern variable, and would therefore be of little taxonomic value. Strains of *Lactobacillus plantarum* showed two to four bands, with great variation in pattern; Rogosa *et al.* (1953) has already suggested that *L. plantarum* as presently constituted, may not be homogenous.

In the study of *Bacillus thuringiensis*, Norris (1964) found a close correlation between esterase types and groups defined by the possession of H antigen. Although the *Streptococcus cremoris* strains used here could be subdivided into four groups based on their phage relationships and on agglutination with immune whey (Whitehead & Bush, 1957; Reiter, Di Biase & Newbould, 1964), there was no difference in esterase pattern among these subgroups. *Leuconostoc mesenteroides* could be divided by esterase pattern into two main groups which closely correlated to the grouping based on physiological tests proposed by Garvie (1960).

The present results indicate that many lactic acid bacteria possess active esterase. It is difficult to verify the 'true esterase', because non-enzymic proteins of milk and of serum (Downey & Andrews, 1965) and some proteolytic enzymes also show esterase activity (Pickering & Reiter, 1962). However, the esterase activities of lactic streptococci and streptobacteria were destroyed by conventional heat tests for enzymes. Inhibition experiment suggests that the activities of *Streptococcus lactis* ML3 and *Lactobacillus casei* C9 might be due to ali esterases.

The main esterase (E_F 59) of *Lactobacillus casei* C7 was found to be a cell-bound enzyme. Liberation of esterase into the medium was negligible with growth in the ordinary culture medium unless the pH was maintained at 6·15 when a weak activity was detected in the supernatant fluid. However, the cell-bound esterase of *L. casei* appeared to be stable, irrespective of the environmental low pH value, because it remained highly active in cells after prolonged incubation, such as 3 to 5 days at 37°.

The rapid decomposition of α-naphthyl acetate by whole organisms of *Lactobacillus casei* as indicated by the immediate development of colour on addition of α-naphthyl

acetate + staining solution suggests that the esterases of *L. casei* might be situated on or near to the cell surface, because it is unlikely that these substances are incorporated rapidly into the organism. The location of the esterase may be just within the cell wall.

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Solidified Media Suitable for the Cultivation of *Clostridium novyi* Type B

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SUMMARY

A solidified medium (NP medium) was devised which supported luxuriant growth of *Clostridium novyi* type B. It consists of peptone, extracts of yeast and liver, blood, glucose and certain other compounds essential as nutrients and/or for poising oxidation-reduction potential. The peptone could be replaced by a commercial preparation of acid-hydrolysed casein or a mixture of 18 amino acids, but the presence of cysteine and either ascorbic acid, thioglycollate (mercapto-acetate) or dithiothreitol (DTT) was essential for consistent rapid and luxuriant surface growth. Blood was not essential in media supplemented with cysteine and DTT, although in its presence the number of colonies which developed was maximal and the formation of zones of haemolysis facilitated the location of smaller colonies. When supplemented with cysteine + DTT several other solidified media, none of which contained yeast—or liver—extract, supported good growth. By incorporating antitoxin into certain clear blood-free plating media containing cysteine + DTT it was possible to show that only some bacilli of a culture produced colonies which developed opaque haloes of toxin antitoxin flocculum. A liquid version of the NP medium was also devised.

INTRODUCTION

Some strains of *Clostridium novyi* will not grow readily on the surface of solid media such as blood agar (Cruickshank, 1965). In our laboratories the serological types A, C and D have been amenable to this mode of culture, but it has been impossible to obtain regular luxuriant surface growth of type B strains on this and many other solidified media, even when supplemented with horse blood, enzyme digest of horse muscle, yeast extract and auto-digest of pancreas. While this has not precluded detection of contaminant organisms it has proved impossible, so far, to recognize and separate variants of *C. novyi* type B of different colonial morphology. For these reasons, and because studies of the biochemical aspects of toxin formation and the nutritional requirements of this important anaerobe (Batty, Buntain & Walker, 1964; Cruickshank, 1965) were contemplated, it was desirable to have available a plating medium which could be made simply and reproducibly and which could be relied upon to support luxuriant growth of *C. novyi* type B.

Organism. Clostridium novyi type B strain CN 755 was selected from the Culture Collection of the Wellcome Research Laboratories. Stock cultures grown in Robertson's meat broth (RMB) for 18–36 hr at 37° were kept subsequently at room temperature and subcultured at monthly intervals

Inocula. When an inoculum was required a subculture from stock was made in fresh RMB and incubated overnight at 37°. Immediately before use the growth was resuspended by gently swirling the culture to form a relatively even suspension of bacteria; this was then left to stand for a few minutes to allow meat particles to settle. Two drops (about 0·06 ml.) of bacterial suspension were transferred to 6 ml. liquid media, and six drops (about 0·18 ml.) to 20 ml. portions. With solidified media a single loopful (3 mm. diameter) of suspension per plate was streaked when comparisons of colonial morphology were to be made. For more critical assessment of the essential nature of components of media or for comparison of different media, single drops (about 0·03 ml./plate) of suspension were delivered from a pipette and spread by a sterile stainless-steel loop (Moore, 1966).

Growth in liquid media. Volumes (6 ml.) of liquid medium were contained in screw capped 13 × 100 mm. Pyrex Culture Tubes (Jobling and Co. Ltd., Sunderland, England); 20 ml. volumes were contained in 1 oz. screw capped bottles (United Glass (England) Ltd., Staines, Middlesex). After aseptic addition of sterile supplements and inoculum, the caps were screwed down tightly and the vessels incubated at 37°.

Growth on solidified media. Sterile plastic Petri dishes (Sterilin, Richmond, Surrey, England) were used. Anaerobic conditions were maintained by the use of modified McIntosh & Fildes jars (Baird and Tatlock, Ltd., Chadwell Heath, Essex, England; see Batty & Walker, 1965). Before use, the insides of the jars were washed with clean warm water and dried, and an extra satchet of palladium catalyst was placed on the bottom of the jar (Irene Batty, personal communication); these satchets were renewed after the jars had been used three or four times. After streaking, plates were placed quickly inside the anaerobic jar. This was evacuated to 0·1 mm. Hg, filled with a mixture of hydrogen and carbon dioxide (95 %, v/v, H_2 + 5 %, v/v, CO_2), re-evacuated and re-filled with more gas mixture. Incubation was at 37°.

Measurement of growth in liquid media. The opacities of cultures were read with an EEL colorimeter and are expressed in terms of extinction at appropriate wavelengths. Since different media varied in colour and absorbancy it was necessary to vary the filters used. For any one experiment the filter chosen was one which allowed adjustment of the instrument to 100 % transmission when blanks of uninoculated media were inserted. The filters used were nos. 47, 49 and 70, which transmit maximally at 465 mμ and 680 mμ, respectively. By use of an adaptor the Pyrex culture tubes could be inserted directly into the instrument thus enabling repeated measurements of opacity *in situ*. With 1 oz. bottles it was most convenient to tip a portion of the culture into a 6 in. × $\frac{5}{8}$ in. bacteriological test tube used as optical cell.

Measurement of growth on solidified media. The amount of growth on plated cultures was assessed by gross appearance and is described as luxuriant, good, fair, poor or nil corresponding to the presence of more than 500; 200 to 500; 50 to 200; 10 to 50 and 0 colonies/plate.

Medium constituents. Yeast extract, Trypticase Soy Agar, Trypticase Soy Broth, Bacto Tryptose, Bacto Tryptone, Neopeptone, Vitamin-free Casamino-acids and agar (Noble) were all Difco products. The liver extract was a dried preparation (Pabryn Laboratories, Greenford, Middlesex). Inorganic salts, glucose, ascorbic acid, cysteine hydrochloride and glutamine were of A.R. quality (British Drug Houses Ltd.). Dithiothreitol (DTT) A grade, was obtained from Calbiochem Ltd. All solutions were made in glass-distilled water.

Sterilization. The basal portion of all media used, containing a nitrogen source, yeast extract (YE), liver extract (LE), glucose and agar, was autoclaved at 121° for 15 min. Solutions of ascorbic acid, cysteine, glutamine and DTT (of unknown thermal stability) were filtered through Millipore GS membranes just before use.

Photomicrography. Colonies and Nigrosin smear-preparations of bacteria were photographed under a Vickers projection microscope.

RESULTS

First attempts to obtain a plating medium used a papain digest of horse muscle (pH 7·60) supplemented with growth factors of the vitamin B complex, glucose and glycyl-L-asparagine (Takarabe, 1960) and was solidified with agar (1 % or 3 %, w/v). Although the liquid cultures, from which inocula were taken, always appeared by phase microscopy to contain numerous viable (non-granular) bacilli, their cultivation on the above solid medium met with only sporadic success.

It was realized that unknown essential factors, possibly of a peptide nature, might be missing, but it was suspected that labile thiol compounds were more probably involved. The loss of these to varying and usually decisive extents each time plates were poured and dried could well account for the variable growth responses observed. Attempts to simulate a solidified version of Robertson's meat broth by inclusion of subsurface meat particles and cysteine in the above medium were attended with success only occasionally. It was difficult to overlay sterile meat particles, placed in a Petri dish, with molten medium, and the final surface was invariably interrupted by protrusion of larger particles. Such a medium was therefore not easy to make or reproduce. Homogenization of the meat particles resulted in complete failure of the medium. Subsequent work showed that maintenance of the agar surfaces in a moist condition was not a factor decisive for growth.

Following the report of Khairat (1966) who described a medium for the cultivation of many fastidious obligate anaerobes, a fresh approach was made to the cultivation of *Clostridium novyi* type B on solidified medium. Khairat stressed the synergistic effect of yeast and liver extracts and attributed much of the success of his medium to this. Commercial preparations of yeast and liver extracts were mixed with agar and glucose and different commercial sources of nitrogen (Bacto Tryptone, Trypticase Soy Agar, Trypticase Soy Broth, Neopeptone) were added singly to form various basal media. Sterile molten portions of each (at pH 7·60) were then supplemented aseptically with horse blood and a sterile solution containing ascorbic acid, cysteine, glutamine and DTT. Plates were quickly poured, dried (15 min., 37°), streaked and then incubated anaerobically. Dithiothreitol was used following the report by Cleland (1964) of its low oxidation-reduction potential (−0·33 V at pH 7·0) and its use for the specific protection of labile thiol compounds. From a qualitative assessment of growth the medium containing Neopeptone was chosen as the best and most economical, and is referred to subsequently as NP medium. Growth of *C. novyi* type B CN 755 on this is shown in Pl. 1, fig. 1.

Composition of liquid media

Omission of horse blood and agar produced liquid NP medium which gave heavy growth of *Clostridium novyi* within 18 hr at 37°. Neopeptone could be replaced by acid hydrolysate of casein (CA medium) or a mixture of eighteen amino acids (Aa medium)

each present at concentrations between 50 and 100 μg./ml. Growth in this latter medium, however, was very poor as compared with growth in NP medium or CA medium. In NP medium a supplement of cysteine only was essential, whereas in CA medium a mixture of cysteine with either ascorbic acid or DTT was essential (Table 1). CA medium did not support such heavy total growth as NP medium so that the essential nature of cysteine in the CA medium was less clearly shown.

Table 1. *Growth of Clostridium novyi type B CN755 in liquid NP and CA media supplemented with ascorbic acid, cysteine and dithiothreitol, separately and in combination*

Supplements: (A) ascorbic acid 250 μg./ml., (C) cysteine 100 μg./ml., (D) dithiothreitol 100 μg./ml. Unwashed inoculum = culture of *C. novyi* grown for 24 hr in Robertson meat broth and then subcultured into liquid NP medium supplemented with cysteine (100 μg./ml.), glutamine (50 μg./ml.), dithiothreitol (100 μg./ml.); pH 7·80; 6 drops (0·18 ml.) transferred to each 20 ml. portion of test medium. Washed inoculum = 5 ml. of the same culture centrifuged aseptically, supernatant phase discarded and bacteria resuspended in 5 ml. of sterile quarter-strength Ringer solution; 2 drops transferred to each 20 ml. portion of test medium. Glutamine (50 μg./ml.) was present in all test systems.

	Basal CA medium		Basal NP medium	
Supplements	Washed inoculum	Unwashed inoculum	Washed inoculum	Unwashed inoculum
A+C+D	0·69*	0·70	0·99	1·01
C+D	0·60	0·56	1·01	1·02
A+D	0·31	0·30	0·28	0·36
A+C	0·60	0·65	1·16	1·12
D	0·0	0·0	0·26	0·25
C	0·0	0·0	1·10	0·97
A	0·0	0·0	0·14	0·17

* Extinctions of cultures measured at 680 mμ in EEL colorimeter with 6 in. × $\frac{5}{8}$ in. bacteriological test tube as optical cell and uninoculated medium as blank. Values shown are the averages of triplicate cultures.

Composition of solidified media

Different concentrations of Neopeptone between 1 and 4 % (w/v) were without obvious effect on growth. It could be replaced by 1 % (w/v) acid hydrolysate of casein (CA medium), or a mixture of amino acids (Aa medium) (Pl. 3, fig. 11, 13, 15) or by crude papain digest of horse muscle (PD medium). Omission of horse blood decreased, to some extent, the number of colonies which developed on plates, but since residual growth was still luxuriant blood was stimulatory rather than essential. In the presence of cysteine and absence or presence of blood, ascorbic acid or DTT could be omitted, separately, without adverse effect on growth. However, the omission of both compounds when cysteine was present, or of cysteine when ascorbic acid and/or DTT was present resulted in development of markedly fewer colonies than on control plates. These colonies had a thin, flat, dry appearance and the fact that they had developed at all was probably due to the presence of traces of essential compounds, possibly thiols, carried over in the inoculum at the time of streaking. Omission of all three compounds resulted in complete absence of growth. Thioglycollate at 100 μg./ml. replaced DTT in this medium but did not do so in CA or Aa media (Pl. 3, fig. 12, 14, 16)

The effect on growth of initial pH value

Growth on solidified NP medium (blood omitted) was equally good at all pH values in the range 6·70 to 8·00. In liquid medium (blood omitted) growth was tested in the range pH 7·60 to 8·50 and was heavy at all these values. At pH 8·50, however, the lag period was increased; growth was poor at 24 hr but heavy at 48 hr.

The effect on growth of drying the surface of the solidified medium

Ten plates of NP medium were poured and allowed to set at room temperature (about 20°). Five of these were then placed in the incubator at 37° and left to dry in an inverted, sloping position, with lids removed, for 15 min. The remaining five plates were left at room temperature with lids on during this time and were subsequently called wet plates. Each plate of the dry and wet series was then streaked with one loopful of a culture of *Clostridium novyi* CN 755 grown at 37° in RMB for 18 hr. The appearance of the colonies which developed after subsequent incubation for 24 hr is shown in Pl. 1, fig. 4, 5. It was clear that whilst the condition of the surface of the medium with respect to 'free' moisture was not a determinant factor for growth it did have a marked effect upon the morphology of the colonies. Free moisture seemed to favour motility with formation of characterless spreading confluent growth.

Preparation of solidified NP medium

The solidified medium (NP medium) finally adopted for the routine plating of cultures of *C. novyi* type B had the composition shown in Table 2. The initial pH of the final medium was between 7·6 and 7·8. Since the presence of blood was not strictly essential for growth it was omitted when its presence would be likely to hinder observation of phenomena other than growth, e.g. formation of toxin antitoxin haloes, or modify certain pre-set conditions, e.g. initial pH value. In the following account the omission of blood is indicated where necessary.

Table 2. *The composition of NP medium used for plating cultures of Clostridium novyi type B CN 775*

	g.		mg.
Neopeptone*	10	Cysteine hydrochloride	100
Yeast extract*	5	Glutamine	50
Liver extract†	5	Dithiothreitol	100
Glucose	10	Horse blood	100 ml.
Agar (Noble)*	20	Salts solution‡	5 ml.

Glass-distilled water to 1,000 ml. pH 7·6–7·8.

* Difco.

† Pabryn Laboratories, Greenford, Middlesex.

‡ Salts solution contained (g./l.): $MgSO_4.7H_2O$, 40; $MnSO_4.4H_2O$, 2; $FeCl_3$ anhyd., 0·4; conc. HCl, 0·5 ml.

Basal medium. To make 1000 ml. of medium the requisite quantities of Neopeptone, yeast extract, liver extract and glucose were dissolved in about 400 ml. glass-distilled water, the salts solution was added, the volume made to 500 ml. and the solution adjusted to pH 7·6 to 7·8. Agar dissolved in 500 ml. distilled water by autoclaving (121° for 5 min.) was then added whilst the solution was still hot. Volumes of 18 ml. were quickly dispensed into 1 oz. screw-capped bottles and sterilized by auto-

claving. When the medium was not used immediately it was stored at 4° and melted when required.

Final plating medium. Liquefied portions of basal medium were allowed to cool to 37 to 40°. Horse blood (2 ml.) was then added followed by 0·15 ml. of a solution (pH 7·6 to 7·8) containing (mg./ml.): cysteine hydrochloride, 12; glutamine, 6; DTT, 12. This solution was made just before use and sterilized by filtration through a Millipore GS membrane. Plates were then poured immediately, allowed to set (about 15 min.) and dried at 37° for not longer than 15 min. Immediately after this the plates were streaked and quickly placed in anaerobic jars which were filled with the H_2+CO_2 mixture. Prepared and used in this way NP medium repeatedly supported rapid (18 hr) growth from the organisms in cultures of *C. novyi* type B grown for 24 hr and 48 hr in RMB. Even after storage of these RMB cultures for 2 or 3 weeks at room temperature many viable organisms were still present. Cultures grown in Brewer's medium for 24 and 48 hr contained many viable organisms but after 72 hr their numbers appeared to be greatly decreased (Pl. 1, fig. 1 to 3).

CA medium. This was essentially the same as NP medium but vitamin-free Casamino acids (1 %, w/v) replaced the Neopeptone; liquid and solidified versions were found to be reliable. Growth could be obtained without horse blood but its presence was beneficial (Table 3). Cysteine with either ascorbic acid or DTT were essential for good rapid growth in liquid and solidified CA media; glutamine could be omitted without adverse effect and glucose could be decreased to as low as 0·12 % (w/v). Above 1 % (w/v) glucose appeared to be unfavourable for growth (Table 3).

Table 3. *The effect of varying some components of solidified NP and CA media on the colonial growth of Clostridium novyi type B CN 755*

Basal media (pH 7·60)	Variation	Growth* No. colonies developed at 18 hr
NP (blood 10 %, v/v; glucose, 1 %, w/v)	Nil	80
	YE†	80
	LE‡ omitted	80
	YE+LE omitted	30
	Glucose at 3 %	20
	Salts§	200
CA (no blood; glucose, 0·12 %, w/v)	Nil	100
	Glucose 1 %	40
	Glucose 3 %	0
	Glucose 1 %+salts§	30
	Glucose 1 %+blood (10 %, v/v)	500

* From inoculum, 1 loopful of 24 hr culture in Robertson's meat broth.
† YE, yeast extract (Difco), 0·5 % w/v.
‡ LE, liver extract (Pabryn), 0·5 %, w/v.
§ In g./l. solution: $MgSO_4.7H_2O$, 40; $MnSO_4.4H_2O$, 2·0; $FeCl_3$, anhyd. 0·4; conc. HCl, 0·5 ml.; 0·1 ml. used/20 ml. medium, and added before adjustment of pH value and subsequent autoclaving.

Miscellaneous solidified media

Following the demonstration of the essential nature of the mixture of cysteine with DTT or ascorbic acid in NP and CA plating media, the suitability for growth of each of four other solidified media was tested. These were: RMB without meat particles

(pH 7·20); RMB without meat particles and supplemented with blood (10 %, v/v); papain digest of horse muscle supplemented with sodium sulphate extract of horse muscle (HME, 10 %, v/v, pH 7·80); Brewers medium without thioglycollate. Each of these media was supplemented, as described for NP medium, with cysteine, glutamine and DTT and solidified with agar (2 %, w/v). Replicate plates of each medium were streaked with 0·03 ml. of a culture of *Clostridium novyi* CN 755 grown in RMB for 24 hr and the plates then incubated at 37° for 60 hr. The colonies and constituent bacilli are shown in Pl. 2, fig. 6 to 10. Smear preparations stained with fluorescent antiserum (Batty & Walker, 1965) confirmed that the rods were those of *C. novyi*. Examination of the whole plates showed that whilst growth was most luxuriant on NP medium it was also good on the other media. No growth occurred on any of these media when the mixture of cysteine and DTT was omitted.

Demonstration of toxinogenic colonies

Clostridium novyi type B antitoxin was incorporated in media at one α-unit/ml. The formation of toxin antitoxin haloes by individual colonies of *C. novyi* CN 755 is shown in Pl. 3, fig. 18. Although the morphology of the four colonies shown in Pl. 3, fig. 17, is identical, comparison of the densities of their toxin antitoxin haloes (Pl. 3, fig. 18) suggests that their toxinogenic capacities were different.

DISCUSSION

The essential need of *Clostridium novyi* type B for the mixture of cysteine with dithiothreitol (DTT) in addition to its need for strict anaerobiosis and for amino acids, components of the vitamin B complex and other unidentified factors (probably peptides) reveals its particularly fastidious character.

In neutral or slightly alkaline solution the oxidation of cysteine proceeds rapidly (Elliott, 1930; Crawhall & Segal, 1966; Leyden & Morgan, 1967) so that under the conditions prevailing during pouring, setting and drying of plating media its loss is likely to be considerable and the final media useless for the cultivation of this organism. It is clear, however, from the work of Cleland (1964) that in admixture with DTT cysteine is protected. Use of cysteine + DTT in the present investigations has resulted in the transformation of apparently useless plating media into media which, being intrinsically complete, are capable of supporting luxuriant growth of *Clostridium novyi* type B. The results suggest that use of cysteine + DTT may provide the key to successful surface cultivation of other fastidious clostridia, and that the mixture might, therefore, be used with advantage in media employed for primary isolation of these bacteria. In plating media thioglycollate is unlikely to be of use for protection of cysteine since both of these compounds are oxidised by air at the same rate (Elliott, 1930). The superior protection of thiols afforded by DTT results from its greater stability to oxidation by air and also from the non-reversible cyclization of an intermediate product, formed by its reaction with disulphides, which ensures the regeneration of thiol (Cleland, 1963).

Neither ascorbic acid nor DTT replace cysteine in solidified medium hence it appears that cysteine is required *per se* and not only as a reducing agent. Szent-Györgi, Együd & McLaughlin (1967) reported that inhibition of bacterial growth by certain keto-aldehydes (C_4—C_6 homologues of methylglyoxal) was instantaneously annulled by adding cysteine because of its ready reaction with these aldehydes. If such

an annullment of inhibition is a determining factor for the growth of *C. novyi* type B in the media used in the present work, the necessity for maintaining cysteine in the thiol form becomes clear.

The unsurpassed success of Robertson meat broth (Willis, 1965) for primary isolation and subsequent maintenance of clostridia probably reflects an adequate content of thiol and all other essential compounds.

The patience, willing co-operation and expertise of both Messrs A. E. Jones and E. J. Kentish who took the photographs is gratefully acknowledged. My thanks are also due to Misses B. Hill and F. Smith for able technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Growth of *Clodstridium novyi* type B CN 755 (culture 79/12) on solidified NP medium (pH 7·80); blood present. Whole plates × 0·8. Bacilli from each type of colony were shown to be *C. novyi* by the fluorescent antibody technique.

Fig. 1. Plate streaked with a culture grown for 24 hr in Brewer medium.

Fig. 1*a*. As fig. 1, but enlarged to show variable morphology of colonies. × 2.

Fig. 2. As fig. 1, but the same culture at 48 hr.

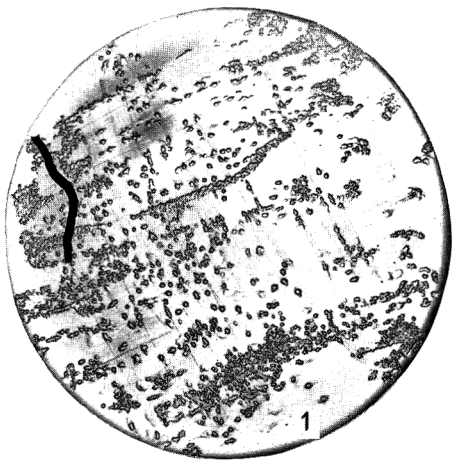
Fig. 2*a*. As fig. 2, but enlarged to show variable morphology of colonies. × 2.

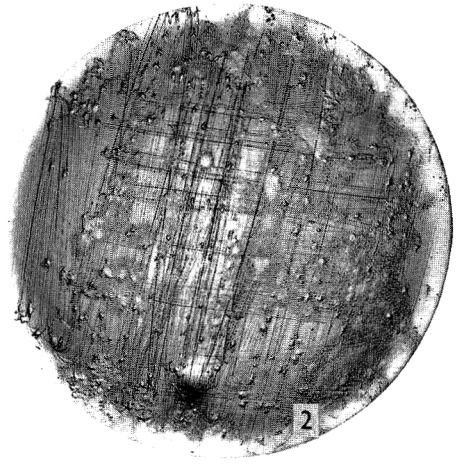
Fig. 3. As fig. 1, but the same culture at 72 hr. There is almost complete absence of viable cells.

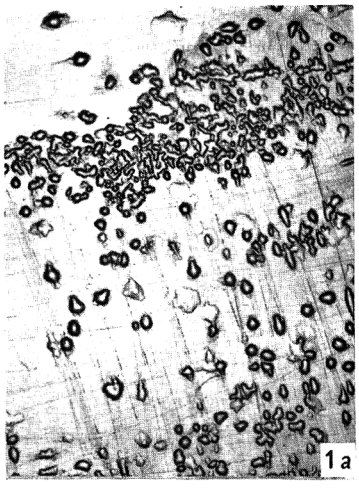
The effect of drying the surface of NP medium on the morphology of colonies of *C. novyi* type B CN 755.

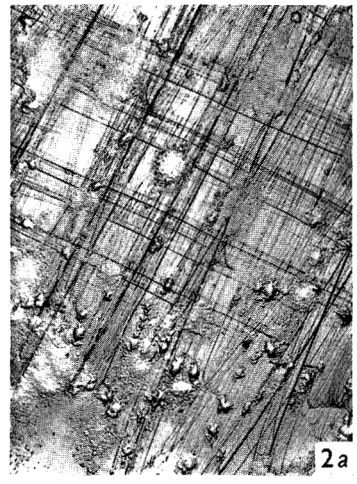
Fig. 4. *C. novyi* CN 755 (culture 68/13) grown at 37° for 24 hr in Robertson meat broth, streaked on a wet plate of NP medium (pH 7·80) and incubated in $H_2 + CO_2$ for 24 hr at 37°. × 6·5.

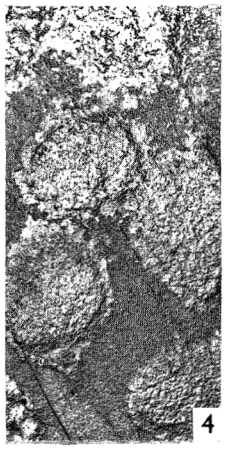
Fig. 5. As fig. 4, but the same culture streaked on to a dried plate of NP medium. × 6·5.

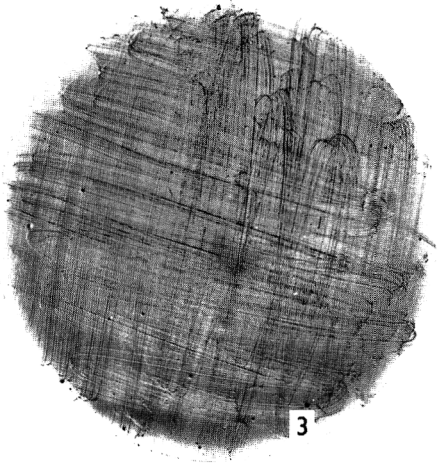












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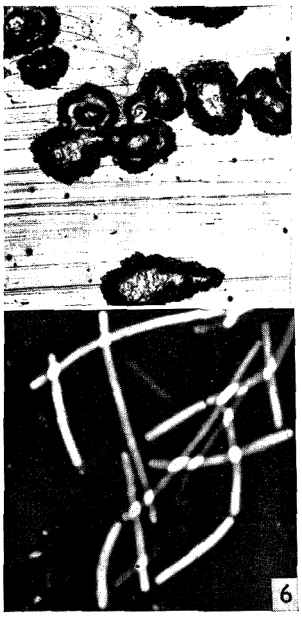

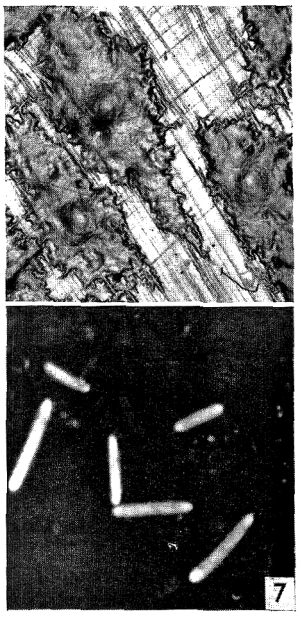

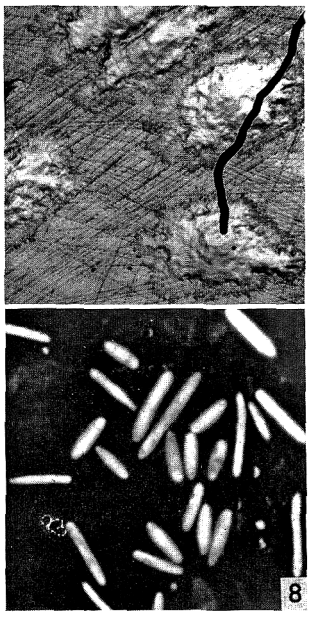

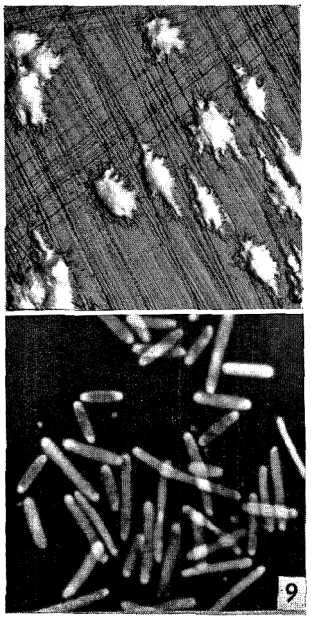

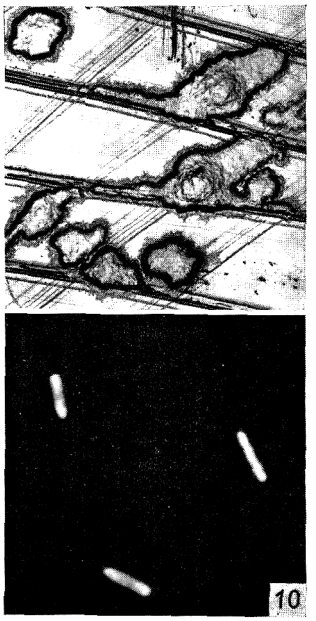

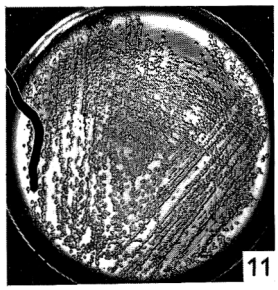
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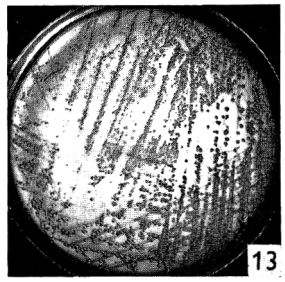
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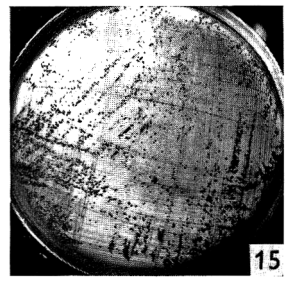
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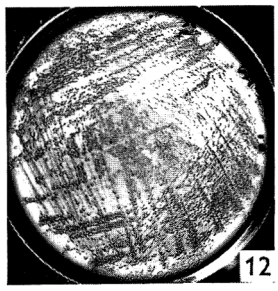
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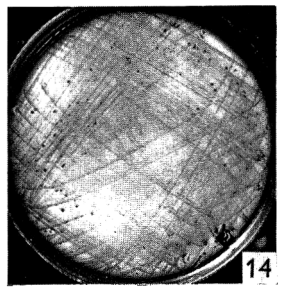
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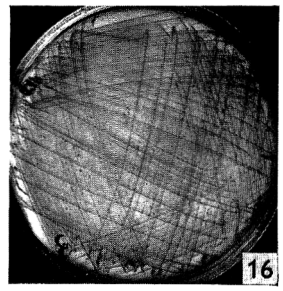
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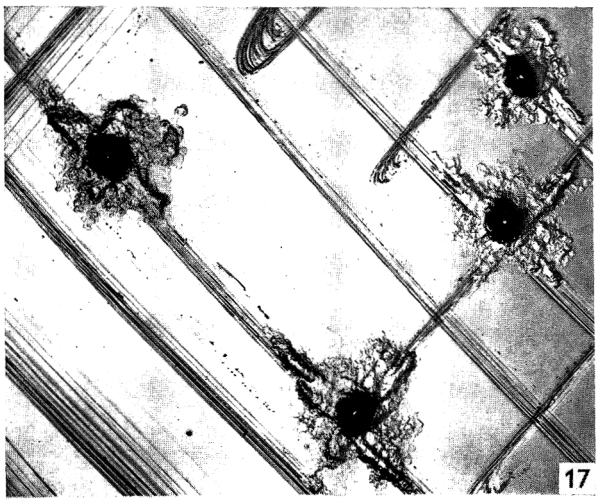
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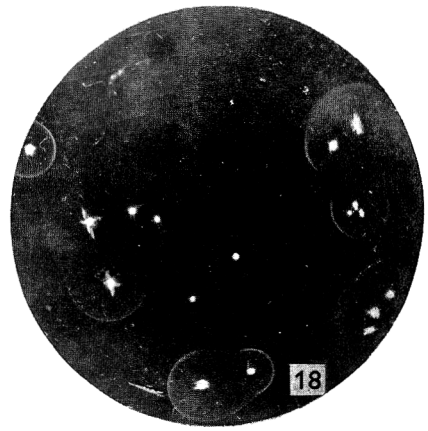
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PLATE 2

Colonial and cellular morphology of *C. novyi* type B CN 755 (culture 83/2) on each of five media supplemented just before use with cysteine, glutamine and dithiothreitol (DTT) (μg./ml.) = 100+ 50+ 100, respectively. An inoculum was grown from a single colony in Robertson meat broth for 24 hr at 37°, 0·03 ml. spread over replicate plates of each medium which were then incubated in H_2+CO_2 for 60 hr at 37°. Bacilli, × 1630; colonies, × 5·5. Bacilli of colonies shown to be *C. novyi* by the fluorescent antibody technique.

Fig. 6. NP medium, blood present (pH 7·80).

Fig. 7. Papain digest of horse muscle containing sodium sulphate extract of horse muscle (10 %, v/v; pH 7·80).

Fig. 8. Robertson meat broth without meat particles (pH 7·20).

Fig. 9. As fig. 8 but supplemented with horse blood (10 %, v/v).

Fig. 10. Brewer medium without thioglycollate, agar at 2 % (w/v).

PLATE 3

Replacement of dithiothreitol (DTT) by thioglycollate. One drop (0·03 ml.) of 24 hr culture of *C. novyi* CN 755 (culture 83/2) grown in Robertson meat broth, spread over the surface of each plate and incubated for 48 hr at 37°. All media supplemented with cysteine (100 μg./ml.) and glutamine (50 μg./ml.); all at pH 7·6–7·8.

Fig. 11. NP medium (blood present) + DTT (100 μg./ml.).

Fig. 12. NP medium (blood present) + thioglycollate (100 μg./ml.).

Fig. 13. CA medium (no blood) + DTT.

Fig. 14. CA medium (no blood) + thioglycollate.

Fig. 15. Aa medium (no blood) + DTT.

Fig. 16. Aa medium (no blood) + thioglycollate.

Fig. 17. Morphology of colonies of *C. novyi* CN 755 (culture 61/16) after growth for 48 hr on Aa medium (no blood) containing cysteine (100 μg./ml.), glutamine (50 μg./ml.), DTT (100 μg./ml.) and *C. novyi* type B antitoxin (one α-unit/ml.). Magnification × 5.

Fig. 18. Whole plants bearing the 4 colonies shown in fig. 17, after storage at 4° for 4 days. Different toxin-producing capacities are suggested by the different densities of the toxin antitoxin haloes. × 0·8.

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Preparation and Some Properties of Active Protoplasts of *Bacillus megaterium*

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SUMMARY

A method for the preparation of protoplasts of *Bacillus megaterium* is described. The protoplasts obtained were very active metabolically and lysates of them were rich in polyribosomes. Methods for the detection of polyribosomes and of nascent peptides are detailed.

INTRODUCTION

A protoplast may be defined as 'a structure derived from a vegetative cell by removal of the entire cell wall, or alternatively that part of the cell which lies within the cell wall and which, in some species, may be plasmolysed away from it' (McQuillen, 1960). It is a consequence of the general difference between the cell walls of Gram-positive and Gram-negative organisms that true protoplasts can only, at present, be obtained from certain Gram-positive species. The methods used to prepare protoplasts and a description of their properties are given in the review of McQuillen (1960). The present method for the preparation of protoplasts was devised so that studies of protein synthesis in *Bacillus megaterium* could be extended to include investigations of the effects of antibiotics upon polyribosomes, which can be obtained from protoplasts by gentle methods (i.e. methods which involve little or no mechanical shearing which would be expected to break polyribosomes). It was also felt that these studies would be more significant if the protoplasts could be obtained in a reproducible vigorously active metabolic state, so that they could be compared with bacteria in the logarithmic phase of growth. Therefore a rapid method for the quantitative conversion of *B. megaterium to* protoplasts was devised; the method is basically similar to that of Yudkin (1963).

METHODS

Cultivation. Lyophilized cultures of *Bacillus megaterium* KM were used to start new stocks at monthly intervals. The organism was maintained by subculturing daily in a liquid medium C (McQuillen & Roberts, 1954) with 0·1 % (w/v) glucose added. For experiments, a basal liquid medium PRE was used. This was similar to medium PR of Yudkin (1963) and contained (g./l.): KCl, 4; $MgCl_2.6H_2O$, 4; NH_4Cl, 2; Na_2SO_4, 0·15; $Na_2HPO_4.12H_2O$, 0·35; glucose, 10; sucrose, 100. Difco Bacto-peptone (0·1 %, w/v) was added when required. Organisms were grown in the basal medium supplemented with peptone at 37° with aeration in the presence of ^{32}P-orthophosphate (15 μC/ml.) for three generations. Under these conditions the extinction at 600 mμ (E_{600}) doubled in 30–35 min.

Preparation of protoplasts. Organisms in the exponential phase of growth, at suspension densities of about 200 μg. dry weight/ml., were harvested by centrifugation (3 min. at 3000 *g*) and resuspended at 10 mg./ml. in the basal medium containing peptone and lysozyme (200 μg./ml.). At 37° the bacilli were quantitatively converted to protoplasts within 2–3 min. It was important that the organisms should be in the logarithmic phase of growth at the time of harvesting, and that harvesting and resuspension were carried out rapidly, otherwise conversion to protoplasts was poor. However, there was no absolute dependence on growth rate. Organisms grown in the absence of peptone (doubling time about 70 min.) were converted equally rapidly, provided they were harvested in the exponential phase of growth. The presence of peptone was not essential during the formation of protoplasts.

Incubation of protoplasts. Immediately after preparation, protoplast suspensions were diluted 50-fold with medium plus peptone (to 200 μg./ml.) and were incubated with shaking at 37°. Important variables here were the size and shape of the vessel used and the volume of suspension in it. Usually 10–15 ml. of protoplast suspension was incubated in a 100 ml. conical flask. During this incubation, the E_{600} of the suspension initially decreased by about 10 % (presumably due to osmotic imbalance across the membrane) and then began to increase exponentially. The time taken for the extinction to double was usually 25–30 min., slightly less than that for whole cells in the logarithmic phase of growth in the same medium. It was also observed that the distribution of polyribosomes in sucrose density gradients changed markedly during this incubation (see later). In no case did the apparent number of protoplasts increase even when the E_{600} doubled; rather the protoplasts increased in size.

Lysis of protoplasts. Samples of protoplasts (usually 0·5 ml.) were pipetted as rapidly as possible, using pre-warmed pipettes, into thin-walled glass vials standing in a metal block in ice. Each vial contained the neutral detergent, Triton X100 and deoxyribonuclease so that after addition of the sample the final concentrations were 0·1 % (v/v) and 5 μg./ml. respectively. In some experiments ribonuclease was also present in the vial (final concentration 5 μg./ml.). After 1 min. in ice, the resultant lysates were fractionated at temperatures not exceeding 4°.

Sucrose density gradient analysis. Linear sucrose density gradients having a total volume of 4·6 ml. were prepared at 3° and were allowed to stand at that temperature for 5–18 hr before use. Each gradient ranged from 15 to 40 % (w/v) sucrose in a buffer containing tris, 0·01 M; magnesium acetate, 0·01 M; KCl, 0·1 M; pH 7·5 at 10° adjusted with HCl, 0·2 ml. of protoplast lysate was layered on each gradient.

Centrifugation was carried out at 2° for 40 min. by using the Spinco SW 39 rotor at 38,000 rev./min. The centrifuge was evacuated for 5 min. before each run. After spinning, the bottom of each tube was pierced with a needle, and successive fractions of 8 drops (about 0·13 ml. total) were collected into tubes containing 1 ml. cold 8 % (w/v) trichloroacetic acid.

The trichloroacetic acid-precipitates were collected on Oxoid membrane filters, rinsed once with 2 ml. 5 % (w/v) trichloroacetic acid and twice with 2 ml. 1 % (v/v) acetic acid, dried at 80° for 30 min. and then counted in a liquid-scintillation counter (Nuclear Chicago Corporation). A toluene-based scintillation fluid containing 4 g. of 2,5-bis-(5′-t-butylbenzoxazolyl-2′)-thiophene (BBOT)/l. was used. Routinely 39 ± 1 gradient fractions were collected. The distribution of ^{32}P from steady-state labelled systems was found to be an accurate indicator of the polyribosome profile and was far

more sensitive than the alternative method of estimating extinction at 260 mμ. Sedimentation coefficients of materials running in the gradients were not directly measured. The term 70S has been used to describe material sedimenting in the same region as the major peak in ribonuclease-treated lysates.

MATERIALS

Deoxyribonuclease (electrophoretically purified) was obtained from the Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Pancreatic ribonuclease (Worthington) was held at 80° for 10 min. before use. Egg-white lysozyme was obtained from Armour Pharmaceutical Co., Ltd., Eastbourne, England. BBOT was obtained from Ciba Ltd. Triton X100 was obtained from The Rohm & Haas Co., Philadelphia, Pa., U.S.A. Actinomycin D (Dactinomycin) was a generous gift from Merck Sharpe and Dohme. Inc., Rahway, N.J., U.S.A. [^{32}P]orthophosphate and [^{3}H]-L-phenylalanine (5 c/m-mole) were obtained from the Radiochemical Centre, Amersham, England.

RESULTS

A suspension of protoplasts was sampled immediately after preparation and later after incubation at 37° for 20 min. by which time the E_{600} was increasing exponentially. Gradient analysis revealed that, typically, about 50 % of the total ribosomal material was present as 70 S monomers initially but that during the incubation there was a

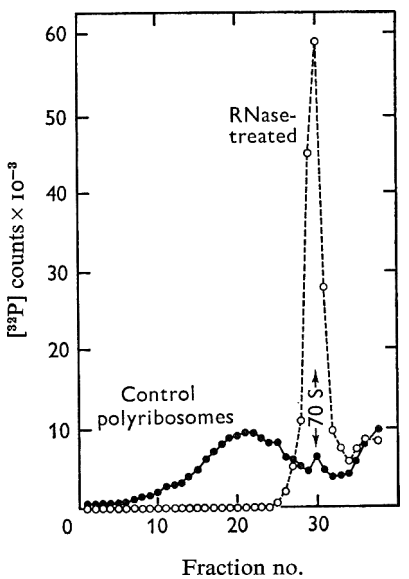

Fig. 1. Polyribosomes from exponentially growing protoplasts: the effect of ribonuclease. [^{32}P]-Labelled protoplasts were lysed during exponential growth. (●) A portion of the lysate was analysed on a sucrose density gradient. (○) Another portion of the same lysate was treated with ribonuclease (5 μg./ml.) for 5 min. at 0° prior to gradient analysis.

decrease in the amount of 70 S material and a corresponding increase in heavier material (polyribosomes). Finally about 85–90 % of the ribosomes were reproducibly recovered as polyribosomes (Fig. 1).

Protoplasts were routinely incubated in this manner prior to commencing each of the following experiments.

Effect of ribonuclease on polyribosomes. Protoplasts were sampled and the lysate was divided into two portions, one of which was treated with ribonuclease as described under Methods. This mild treatment with ribonuclease completely removed all

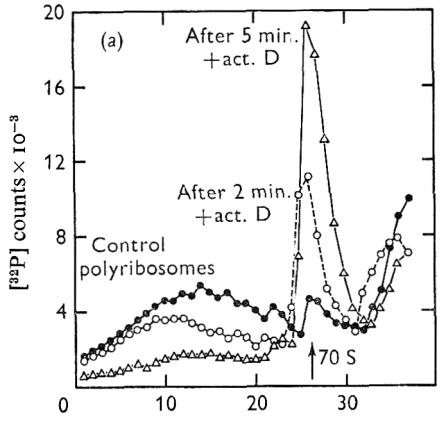

Fig. 2a

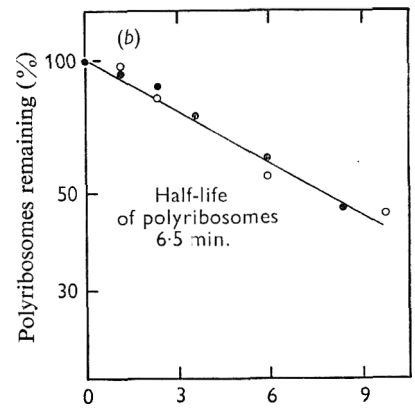

Fig. 2b

Fig. 2a. The effect of actinomycin D on polyribosomes. Actinomycin D (10 μg./ml.) was added to a suspension of [32P]-labelled protoplasts in the exponential phase of growth and the incubation was continued. Samples were taken at intervals and the subsequent lysates were analysed on sucrose density gradients. (●) The protoplasts were sampled immediately before the addition of actinomycin. Other samples were taken 2 min. (○) and 5 min (△) after the addition of actinomycin.

Fig. 2b. Rate of breakdown of polyribosomes in the presence of actinomycin D. Data from duplicate experiments identical to that described in Fig. 2a. Percentages of polyribosomes remaining were computed as described in the text.

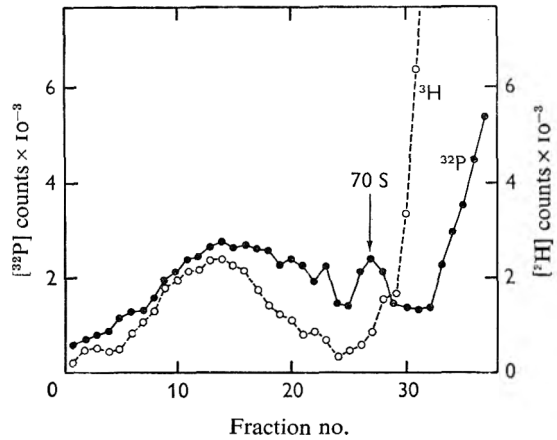

Fig. 3. Nascent protein and polyribosomes. [32P]-Labelled protoplasts were incubated into the phase of exponential growth then [3H]phenylalanine (5 c./m-mole) was added (10 μc./ml.) for 60 sec. Then the protoplasts were sampled and the lysate analysed on a sucrose density gradient (●) [32P]-Labelled polyribosomes, ribosomes, etc. (○) [3H]-Labelled peptides.

the polyribosomes giving a massive peak of monomeric ribosomes (Fig. 1). The extreme sensitivity of the polyribosomes to ribonuclease is in accordance with current ideas concerning the structure of polyribosomes. Also it is obvious from Fig. 1 that some of these structures were very heavy and by assuming that 100 % of the ribosomes present in the 70 S peak after ribonuclease treatment, it was calculated that at least 85 % of the ribosomes had been present as polyribosomes. This is a greater proportion than is usually found in bacterial lysates (see however Flessel, Ralph & Rich, 1967).

Effect of actinomycin D on polyribosomes. Protoplasts were sampled and then actinomycin D (10 μg./ml.) was added to the remainder, incubation was continued and samples were taken at various times. Actinomycin caused a breakdown of polyribosomes with accumulation of ribosomes (Fig. 2*a*). The percentage of ribosomes present as polyribosomes was calculated for each time point as described above and the numbers obtained were expressed relative to the initial value. Figure 2 (*b*) shows that the decay of polyribosomes exhibited approximately exponential characteristics with a half-life of about 6·5 min.

Nascent protein and polyribosomes. Protoplasts were incubated with [^{3}H]phenylalanine (10 μC/ml.) for 60 sec. and a sample was taken for lysis and gradient analysis. Figure 3 shows that [^{3}H]-labelled-material was recovered in the polyribosome region and at the top of the gradient in the 'soluble' region. The specific activity [^{3}H]/[^{32}P] was greatest in the region where the large polyribosomes sedimented, with a minimum at 70 S. This result is in agreement with the idea that nascent peptides are formed on polyribosomes and not on free 70 S ribosome-monomers.

DISCUSSION

A method was developed for the rapid and quantitative conversion of *Bacillus megaterium* to protoplasts. After incubation the protoplasts could be obtained in an active metabolic state and lysates of such protoplasts were rich in polyribosomes. Nascent protein was found to be associated with polyribosomes rather than with free 70 S monomers in keeping with current ideas concerning the mechanism of protein synthesis. Various methods have been developed whereby bacteria may be rendered sensitive to lysis by detergents or by osmotic shock (e.g. Schaechter, Previc & Gillespie, 1965; Flessel *et al.* 1967; Mangiarotti & Schlessinger, 1967). However, some of these methods involve the use of lysozyme, which does not convert drug-inhibited organisms to protoplasts and gentle lysis of such organisms is not usually possible.

Therefore it is considered that protoplasts offer an advantageous system for the study of protein synthesis *in vivo* since they can be lysed promptly and gently to yield undegraded polyribosomes still bearing nascent peptides. Studies of this kind involving antibiotics and protoplasts have been reported elsewhere (Cundliffe, 1967*a*, *b*; Cundliffe & McQuillen, 1967*a*, *b*).

The work described in this paper has been presented in abstract form (Cundliffe, 1967*c*) and as part of a dissertation (Cundliffe, 1967*d*).

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