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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

THE PREPARATION OF PAPERS

'Easy writing's curst hard reading.'—*Richard Brinsley Sheridan.*

'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words.

(2) A paper should be written only when a piece of work is rounded-off. Authors should not be seduced into writing a series of papers on the same subject *seriatim* as results come to hand. It is better, for many reasons, to wait until a concise and comprehensive paper can be written.

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A paper should be submitted in double-spaced typing (top copy) with a 1½ in. left-hand margin, and on paper suitable for ink corrections. The paper should be written

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References. References in the text are cited thus: Brewer & Stewer (1942), (Brewer & Stewer, 1942). Where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made in the text, e.g. (Brewer, Stewer & Gurney, 1944), and subsequently as (Brewer *et al.* 1944); but papers with more than four authors may be cited, e.g. (Cobley *et al.* 1940) in the first instance. Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and the bibliography by the letters *a*, *b*, etc. following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

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Description of Solutions. The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in correct sense, i.e. g./100 g. of solution. For 'per cent of volume', i.e. ml./100 ml., the term '% (v/v)' should be used, and for weight of a substance in 100 ml. of solution, the term '% (w/v)'.

Proprietary Substances and Materials. At first mention, the correct designation of a manufacturer should be given in the text, with address to enable other workers to obtain the product mentioned.

Nomenclature of Amino Acids and Vitamins. The rules published in the *Biochemical Journal* (1952), **52**, 1–2, should be followed.

Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

MICROFUNGI. *Ainsworth & Bisby's Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl, 1–78.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

CORRIGENDA

In Bojalil, L. F., Cerbon, J. & Trujillo, A. (1962).
J. Gen. Microbiol. **28**. 333–346.

Insert on page 335.

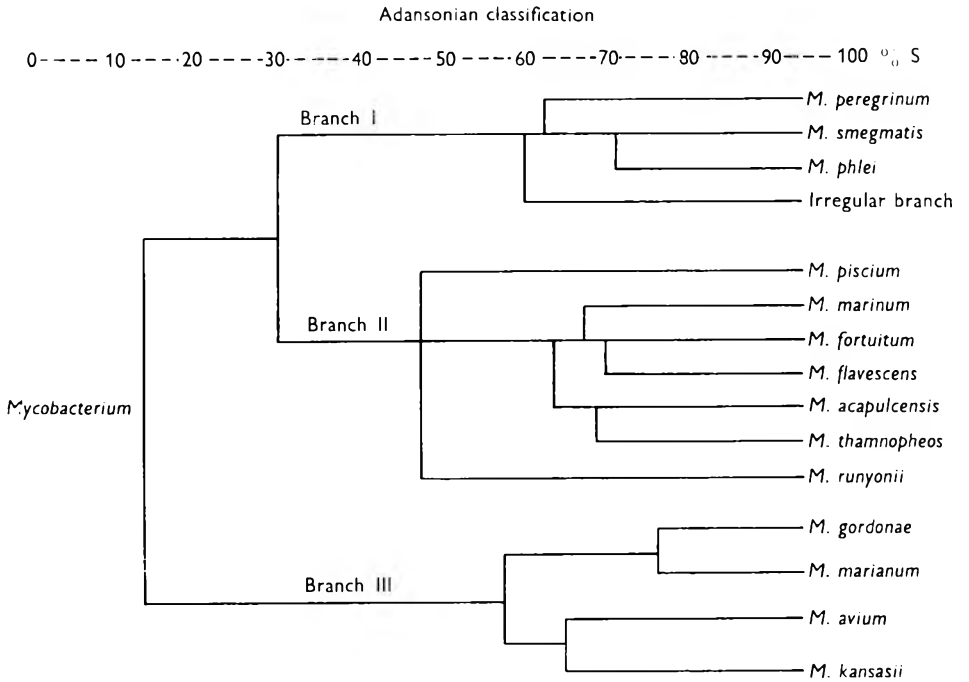

Fig. 2. Taxonomic tree of the mycobacteria. Branch I, high metabolic capacity; Branch II, intermediate metabolic activity; Branch III, low metabolic activity.

On page 342, *for* Fig. 2 *read* Fig. 3.

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Studies on Marine Flavobacteria

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SUMMARY

Sixty-two pigmented strains, 61 of which had been classified as *Flavobacterium* species by the workers who isolated them, were studied.

Morphological, cultural, environmental, biochemical and nutritional studies confirmed that 32 non-motile strains were *Flavobacterium* species; 21 strains were reclassified as presumptive *Cytophaga* species and the remaining 8 strains were ascribed to the genera *Pseudomonas*, *Vibrio* or *Corynebacterium*, or were unclassifiable. A new genus is suggested for the peritrichous flagellated organisms, previously included in the genus *Flavobacterium*. Both the flavobacteria and the cytophagas were found to have many properties in common. Satisfactory methods of differentiating between representatives of these genera were limited to the swarming ability and greater heat resistance of the latter. Of the 32 non-motile *Flavobacterium* strains, 18 were divisible into two well defined groups (? species) and the remainder were a heterogeneous collection. The 22 *Cytophaga* strains were also divisible into two groups (? species). The possibility of a relationship existing between flavobacteria and cytophagas is discussed briefly.

INTRODUCTION

The genus *Flavobacterium* is one of the more commonly represented genera in the marine environment; flavobacteria are normally found in the slime and intestines of fresh and spoiling fish. They have also been isolated from sea water, marine muds and seaweed (for reviews see: ZoBell, 1946; Shewan, 1949; Tarr, 1954). The definition of the genus has undergone considerable modification since it was erected in the first edition of *Bergey's Manual* (1923), where the genus included motile peritrichous- or polar-flagellate, and non-motile Gram-positive and Gram-negative rods. The polar-flagellate forms were removed in the fifth edition of *Bergey's Manual* (1939). The exclusion of the Gram-positive species was suggested by Gary (1950) and Weeks (1955). The genus, as now defined by Weeks (*Bergey's Manual*, 1957), consists of Gram-negative rods, motile by means of peritrichous flagella or non-motile, and producing yellow to brown pigments. However, this definition is not accepted by all workers. For example, Brisou, Tysset & Vacher (1959) gave details of two flavobacteria which were motile by means of polar flagella and Ferrari & Zannini (1958) concluded that the exclusion of the Gram-positive strains from the genus was not justified.

It is also recognized that confusion might arise between the Flavobacterium species and certain yellow-pigmented Cytophage species. Thus Stanier (1947), working with chitin-decomposing cytophagas, showed that they may simulate

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eubacteria under certain circumstances. He considered that many ill-defined eubacterial genera containing Gram-negative rods which do not form spores and are not motile, may contain cytophagas. Gibson (1955) isolated organisms from soil identical with typical cytophagas except in their failure to show gliding motility, and confirmed that the separation of cytophagas from eubacteria was under suspicion. It is known that Cytophaga species are widely distributed in marine environments (Humm, 1946; Starr & Ordal, 1953; Bachmann, 1955; Kadota, 1956; Velankar, 1957), though isolations from fish have been very rare (Shewan & Bain, private communication; Georgala, 1957). It seems possible that the paucity of references to Cytophaga species being found on fish may well be due to the unfavourable conditions under which they have been studied and that many organisms ascribed to the genus *Flavobacterium* should be ascribed to the genus *Cytophaga*. The purpose of the present investigation was to study marine bacteria which had been identified as flavobacteria and determine whether any had been wrongly named.

METHODS

Source of strains. With one exception all 62 strains studied had been identified as Flavobacterium species by the workers who first isolated them; the sources of these strains are given in Table 1. All 62 strains have been deposited in the National Collection of Marine Bacteria (NCMB), Torry Research Station, Aberdeen.

Organisms were maintained on slopes of sea-water agar of the following composition (%, w/v): Lab-Lemco, 1·0; Evans peptone, 1·0; Difco agar, 1·5; aged sea water + distilled water (3 + 1, by vol.). This and all subsequent media were adjusted to pH 7·2–7·3 unless otherwise stated. Strains were subcultured every 4 months.

Flavobacterium aquatile Windle–Taylor strain F 36, the type species, was obtained for comparative purposes; efforts to obtain *Cytophaga hutchinsonii*, type species, or any other authentic named species from Type Culture Collections failed.

Table 1. *Sources of Flavobacterium isolates*

The NCMB number refers to the reference number of these isolates in the National Collection of Marine Bacteria.

NCMB no.	No. of strains	Source	Supplier
244–276	33	Slime of North Sea cod	D. L. Georgala
277–290 and 295	15	Slime or gills of North Sea skate or lemon sole	J. Liston
291–294	4	Lingcod or sea water along the Pacific Coast, near Vancouver	R. A. MacLeod
296–300	5	Fish caught off Iceland	V. Aschehoug
408–411	4	Marine mud or sea water off Californian Coast	C. E. ZoBell
11*	1	Water off Florida Coast	T. J. Starr

* This strain was supplied as a presumptive *Cytophaga* species.

Morphological and cultural characteristics. Organisms were stained by Gram's method after incubation for 4 days at 20° on sea-water agar and their size was measured on similar cultures stained with methylene blue; colony appearances were noted at 5 days. Five-day cultures in sea-water broth (%, w/v: Lab-Lemco, 1·0;

Evans peptone, 1·0; aged sea water + distilled water, 3 + 1, by vol.) incubated at 20° were studied under phase contrast and the appearance of 7-day cultures recorded. Isolates were examined for motility in sea-water tryptone water (Difco tryptone, 1·5 %, w/v; aged sea water + distilled water, 3 + 1, by vol.). Only motile strains were stained for flagella; the technique was based on a method employing Kirkpatrick's fixative and Casares–Gil's modification of Plimmer & Paine's flagella stain (*Staining Procedures*, 1947). Motile organisms were also examined with the electron microscope.

Effect of environmental conditions on growth

A standard inoculum for all tests was one loopful of a 5-day sea-water broth culture.

Temperature range of growth. The ability of the strains to grow on sea-water agar at 37°, 30°, 25°, 20°, 10°, 5° and 0° was noted, the incubation periods extending from 1 to 6 weeks, depending upon the temperature of incubation.

Heat resistance. The viability of cultures grown in 3 ml. of sea-water broth for 5 days was investigated by heating for various times (15, 30, 45, 60 min.) at 45° and 55° before plating the heated cultures on sea-water agar and incubating at 20° for 10 days. Those isolates which failed to grow at 37° were investigated for ability to withstand this temperature by inoculating them on two series of sea-water agar plates. One set was incubated at 37° for 24 hr. and the other for 48 hr.; both were then incubated for a further 7 days at 20°.

Anaerobic growth. Organisms were incubated anaerobically on sea-water agar in McIntosh & Fildes jars for 14 days at 20°.

Salinity range of growth. The strains were plated on Oxoid blood agar base, with additional NaCl (Analar) where required, to give final NaCl concentrations (%, w/v) of 0·5, 2·0, 4·0, 6·0 and 10·0; they were then incubated for 14 days at 20°.

Sensitivity to antibiotics and a 'vibriostatic' compound. The isolates were tested for sensitivity to penicillin (2·5 i.u. per tablet), streptomycin (80 μg.), chloramphenicol (100 μg.), terramycin (10 μg.) and a vibriostatic compound (2,4-diamino-6,7-di-*iso*propylpteridine) as described by Shewan, Hodgkiss & Liston (1954). Standard agar plates, prepared as for sea-water agar but with distilled water + 0·5 % (w/v) NaCl were used, since sensitivity to antibiotics is markedly different on sea-water agar, the higher salt concentration either masking the inhibitory effects of the antibiotics or stimulating bacterial growth.

Physiological tests

The same standard inoculum was used as previously. All the media were incubated at 20°.

Gelatin liquefaction. Nutrient gelatin medium (%, w/v: Lab-Lemco, 1·0; Evans peptone, 1·0; NaCl, 2·0: Oxoid gelatin, 12·0) was inoculated and incubated for 6 weeks, the appearance and extent of liquefaction being noted at regular intervals.

Action on litmus milk. The cultures were incubated in litmus milk for 6 weeks, changes in appearance being noted at regular intervals.

Reduction of nitrate to nitrite. Nitrite was tested for by the Griess–Ilosvay reagents after incubation for 10 days in nitrate broth (%, w/v: Lab-Lemco, 1·0;

Evans peptone, 1·0; NaCl, 2·0; KNO_3, 0·1). Powdered zinc was used to test for false negatives.

Ammonia production was tested for by Nessler's reagent after incubation for 10 days in sea-water peptone water (Evans peptone, 1·0%, w/v; aged sea water + distilled water, 3 + 1, by vol.).

Hydrogen sulphide formation was detected with strips of filter paper impregnated with lead acetate on cultures incubated for 14 days in sea-water broth containing 0·01% (w/v) cysteine hydrochloride (final pH 7·6).

Indole formation was determined by Ehrlich's reagent after xylene extraction of cultures incubated for 10 days in sea-water tryptone water.

Hydrolysis of urea. Cultures were incubated for 14 days on slopes of Christensen agar (1946), modified by the inclusion of (%, w/v): KH_2PO_4, 0·05; K_2HPO_4, 0·05; Oxoid yeast extract, 0·01; bromthymol blue, 0·003; aged sea water + distilled water (3 + 1, by vol.). To ensure that the alkalinity produced was due to urea hydrolysis a control medium without urea was inoculated with the urease-positive strains. Only those strains which gave a markedly more alkaline reaction in the urea medium were considered to be true positives.

Trimethylamine oxide reduction. Cultures were incubated for 14 days in Wood & Baird's medium (1943) modified by the use of aged sea water + distilled water (3 + 1, by vol.). The presence of trimethylamine was then tested for according to the method given in *Topley and Wilson's Principles* (1955).

Production of acid from carbohydrate. Cultures were incubated for 28 days in a medium composed of: Evans peptone, 0·5% (w/v); NaCl, 2·0% (w/v); Andrade's indicator, 1·0% (v/v); test carbohydrate, 1·0% (w/v). Each culture was examined regularly for acid and gas production. The following carbohydrates were used: arabinose, xylose, rhamnose, glucose, fructose, mannose, galactose, sucrose, maltose, lactose, trehalose, raffinose, starch, inulin, mannitol, dulcitol, sorbitol, inositol, salicin.

Glucose utilization. The method of Hugh & Leifson (1953) was used to determine whether glucose was used oxidatively or fermentatively.

Production of 2-ketogluconic acid. Cultures were incubated for 7 days in Paton's medium (1959). All the strains which grew in this medium were examined for 2-ketogluconic acid production by using the reagent of Hough, Jones & Wadman (1950).

Starch hydrolysis. After incubation of the strains in the starch medium for 28 days (see *Production of acid from carbohydrates*) those cultures which showed no acid production were tested with a drop of iodine solution for the presence of starch breakdown products.

Tributyrin hydrolysis. Cultures were incubated and examined on Oxoid tributyrin agar for 14 days.

Growth in Koser's citrate medium was read after incubation for 7 days; strains which showed growth after two serial subcultures were recorded as positive.

Voges–Proskauer and methyl red tests. Cultures were tested after incubation for 10 days in glucose phosphate broth by Barritt's modification for acetylmethylcarbinol (*Topley and Wilson's Principles*, 1955).

Digestion of chitin. Chitin, prepared from crab shells by the method of Benton (1935), was incorporated in the following medium (%, w/v): chitin, 3·5; glucose,

0·1; Evans peptone, 0·1; K_2HPO_4, 0·005; Difco agar, 1·5; aged sea water + distilled water (3 + 1, by vol.). Cultures were incubated for 6 weeks and examined at regular intervals.

Decomposition of cellulose. Cultures were incubated for 6 weeks on a medium consisting of (%, w/v): Whatman powdered cellulose, 5·0; Oxoid yeast extract, 0·02; Evans peptone, 0·2; KH_2PO_4, 0·1; $FePO_4.2H_2O$, trace; Difco agar, 1·5; aged sea water + distilled water (3 + 1, by vol.). The plates were examined at regular intervals.

Blood agar. The cultures were incubated on horse-blood agar for 14 days and examined regularly for growth and haemolysis.

Actomyosin agar. Actomyosin, prepared from fish muscle by the method of Connell (1958), was incorporated in normal sea-water agar at 1·0 % (w/v), giving an opaque medium. The cultures were incubated for 6 weeks and examined regularly for actomyosin breakdown as indicated by clear zones.

Catalase and oxidase formation. Catalase was tested for by adding hydrogen peroxide (20 vol.) to 3-day cultures on sea-water agar; for oxidase formation the method of Kovacs (1956) was used on similar cultures.

Nutrition

Preparation and cultivation of inoculum. The standard inoculum for all tests was one loopful of a 5-day sea-water broth culture. Media were incubated for 7 days at 20° and only those strains which showed visible growth after two serial subcultures were recorded as positive.

Media preparation. The compositions of the media used are given in Table 2. All media were adjusted to pH 7·2–7·3 and sterilized by intermittent steaming.

Swarming

A preliminary study with different concentrations of agar and peptone indicated that swarming was most markedly dependent on the peptone concentration with 2 % (w/v) agar. Therefore the basal medium consisted of (%, w/v): Lab-Lemco, 0·1; Difco agar, 2·0; aged sea water + distilled water (3 + 1, by vol.). To this were added different amounts of Evans peptone as follows (%, w/v): 8·0, 4·0, 1·0, 0·25, 0·05 and 0·01. The Lab-Lemco was included to stimulate growth; without it colonies were so minute on the lower peptone concentrations as to be impossible to be examined visually for swarming. The Lab-Lemco appeared to exert no inhibiting effect on swarming. After the plates had been poured with these media they were dried for 30 min. at 55° and then stored overnight at 20°, before inoculation with the standard inoculum. The plates were incubated at 20° for 10 days when colony appearance and any indication of swarming was noted. *Flavobacterium aquatile* F 36 was plated on distilled water media containing 0·5 % (w/v) NaCl and the lowest three peptone concentrations.

Cell-wall studies

Staining of cell walls. Cell walls were stained by using the phosphomolybdic acid mordant technique recommended by Hale (1953).

Cell-wall analysis. Strains NCMB 244, 249, 251 and 253 were taken as representatives of Groups 1–4 (see Results). *Flavobacterium aquatile* F 36 and strain NCMB 11 were used as controls for the genera *Flavobacterium* and *Cytophaga*, respectively.

Table 2. *Composition of media*

		No. medium					
		1 Basal medium	2 Amino acid medium	3 Growth factor medium	4 Growth factor medium	5 Purine + pyrimidine medium	6 Peptone medium*
Basal constituents	(g./l.)						
Glucose	5	+	+	+	+	+	+
NaCl	25	+	+	+	+	+	+
K_2HPO_4	1	+	+	+	+	+	+
$MgSO_4.7H_2O$	1	+	+	+	+	+	+
$(NH_4)_2SO_4$	2	+	+	+	+	+	+
$CaCl_2$ (anhyd.)	trace	+	+	+	+	+	+
$FeCl_3.6H_2O$	trace	+	+	+	+	+	+
Amino acids, each at 100 mg./l.: glycine, DL-alanine, DL-serine, L-cysteine, DL-threonine, DL-methionine, L-valine, DL-leucine, DL-phenylalanine, DL-tyrosine, DL-tryptophan, L-histidine HCl, DL-ornithine HBr, L-arginine, DL-glutamic acid, DL-aspartic acid, DL-proline		−	+	+	+	+	+
Growth factors (μg./l.) *p*-aminobenzoic acid (100), nicotinic acid (500), pantothenic acid (500), pyridoxine HCl (200), thiamine HCl (500), riboflavin (100)		−	−	+	+	+	+
Folic acid (1), biotin (2), β-alanine (500), vitamin B_{12} (0·5)		−	−	−	+	+	+
Purines + pyrimidines, each at 200 mg./l. uracil, adenine, guanine, xanthine		−	−	−	−	+	+
Evans peptone (1 g./l.)		−	−	−	−	−	+

* Filtered sea water was added to the peptone medium (6), in the ratio medium (6): sea water (3:1), giving an additional medium (7).

+, Constituent(s) included in medium. −, Constituent(s) absent.

Culture media. The same method was used for all isolates except that *F. aquatile* was grown in standard nutrient broth whereas the marine bacteria were grown in sea-water broth. Three hundred ml. amounts of media were used in 1 l. conical flasks. After inoculation, the flasks were shaken for 3 days on a Townson and Mercer

'lateral' shaker at a speed of 80–90 throws/min., the incubation temperature being 20°.

Preparation and hydrolysis of cell-wall suspensions. The technique used was that of Cummins & Harris (1956).

Chromatography. For amino acids and hexosamines, two-dimensional ascending chromatograms on Whatman No. 1 paper were used. Less cell-wall material was available with strains NCMB 244 and 249 because of the comparative difficulty in centrifuging them; this effect was frequently noticed when centrifuging marine organisms grown in sea-water based media. Phenol + water (80 + 20, by vol.) in an ammoniacal atmosphere was used as the first solvent and lutidine + collidine + water (150 + 100 + 120, by vol.) as the second. The spots were located by spraying with ninhydrin solution.

Sugars were detected by descending chromatography on Whatman No. 1 paper with ethyl acetate + pyridine + water (120 + 50 + 40, by vol.) as solvent and aniline hydrogen phthalate (Partridge, 1949) as locating agent; because of similar R_F values arabinose and mannose were distinguished by placing the chromatogram under the ultraviolet light, when the arabinose fluoresced and the mannose appeared as a brown spot.

RESULTS

Sixty of the strains studied were Gram-negative, of which 54 were non-motile and 6 motile; the remaining 2 strains were Gram-positive and non-motile. An analysis of the results obtained with the 54 non-motile Gram-negative bacteria clearly suggests that many of these organisms could be placed in one of the following 4 main groups.

Group 1. Usually small slender rods producing pale yellow or yellow-green pigmented colonies on agar. Growth range 0°–30°. Resistant to 45° for 1 hr. and to 37° for 48 hr. Markedly sensitive to penicillin and to the 'vibriostatic' compound. Gelatin liquefied slowly, if at all; litmus milk unchanged. Ammonia not formed from peptone. Carbohydrates attacked with production of acid; lactose and starch not attacked. Generally complex nutritional requirements, failing to grow on defined media. This group is composed of strains NCMB 244–6, 248, 252, 255, 258–9, 261, 263, 273, 281, 295 and 298.

Group 2. Usually short, oval rods but some pleomorphic. Deep yellow or orange-yellow pigmented colonies. No growth at 30°. Not resistant to 45° for 1 hr. or to 37° for 24 hr. Gelatin liquefied. Litmus milk reduced, with peptonization (NCMB 267, 270–1) or with an alkaline reaction (NCMB 260, 262, 265, 269, 284). Carbohydrates not attacked and starch not hydrolysed. Complex nutritional requirements but grow on defined media. No growth on blood agar. This group is composed of strains NCMB 250–1, 260, 262, 264–5, 267–72, 276, 284 and 289.

Group 3. Short oval or pleomorphic rods. Deep yellow or orange-yellow pigmented colonies. No growth at 30°. Not resistant to 45° for 1 hr. but viable after 48 hr. at 37°. Gelatin not liquefied. Litmus milk reduced. Most carbohydrates not attacked, but starch hydrolysed. Nutritional requirements not complex and growth factors not required. This group is composed of strains NCMB 249, 254 and 287.

Group 4. Long slender rods, pleomorphic. Pale yellow or yellow-green pigmented colonies. Not resistant to 45° for 1 hr. but viable after 48 hr. at 37°. Litmus milk

reduced. Carbohydrates attacked, including lactose and starch, with production of acid. Complex nutritional requirements but will grow on defined media. This group is composed of strains NCMB 247, 253, 256–7, 275 and 285.

Thirty-eight isolates are included in the 4 groups above. Of the remainder, strain NCMB 11 is confirmed as a Cytophaga species and strain NCMB 292 is also considered to be a typical cytophaga. Both these organisms swarmed readily on normal media and, in addition, had the following properties in common with the genus *Cytophaga* (*Bergey's Manual*, 1957): (1) morphology, being long, slender rods with axes straight, bent, U- or S-shaped; (2) absence of microcysts; (3) yellow pigmented spreading colonies typical of cytophagas. Other properties these organisms had in common were: (4) oxidase-positive; (5) liquefaction of gelatin; (6) resistance to 45° for 1 hr.; (7) viability after 48 hr. at 37°. Neither is described in the genus *Cytophaga* in *Bergey's Manual* (1957).

The remaining 14 Gram-negative non-motile strains cannot be justifiably placed in any of the above groups. An analysis of their properties suggests that further small groups may be formed but these groups would be less rigid in their definition than those already described. Three strains, NCMB 282, 291 and 408, appear to merit individual species rank, each being markedly different from any other organisms.

The 6 Gram-negative motile bacteria formed a heterogeneous collection as follows.

(1) Strain NCMB 283 is considered to be a Vibrio species because it: (*a*) is motile with a single polar flagellum; (*b*) produces acid in glucose; (*c*) is fermentative in Hugh and Leifson's medium; (*d*) is sensitive to the vibriostatic compound.

(2) Strain NCMB 286 is considered to be a pigmented Pseudomonas species, failing to produce acid from carbohydrates, because it: (*a*) is motile with polar flagella; (*b*) reduces nitrate to ammonia or nitrogen; (*c*) is oxidase-positive; (*d*) reduces trimethylamine oxide. However, it is penicillin-sensitive, a rare property in the genus.

(3) Strain NCMB 294 is considered likely to be a pigmented Pseudomonas species, failing to produce acid from carbohydrates, because it is: (*a*) motile with polar flagella; (*b*) oxidase-positive; (*c*) insensitive to penicillin.

(4) Strain NCMB 297 is a pink-pigmented peritrichously flagellate organism producing acid from several carbohydrates including glucose and lactose; it cannot be satisfactorily ascribed to any genus.

(5) Strains NCMB 296 and 300 are grouped together because they have many properties in common including: (*a*) yellow-green pigmented colonies; (*b*) peritrichous flagella; (*c*) resistance to 45° for 1 hr.; (*d*) strong hydrogen sulphide production; (*e*) strongly urease-positive; (*f*) reduction of nitrate to nitrite; (*g*) no liquefaction of gelatin; (*h*) growth in basal medium; (*i*) production of 2-ketogluconic acid; (*j*) hydrolysis of tributyrin. According to *Bergey's Manual* (1957) they would be identified as Flavobacterium species but they are not similar to any species listed.

The Gram-positive strains, NCMB 280 and 299, are from their morphology, colony appearance and biochemical reactions, typical Corynebacterium species.

The characters distinguishing many of the strains have been mentioned already; detailed properties of individual strains are omitted for brevity but the principal results are summarized below.

Morphological and cultural characteristics

Gram reaction. With the exception of the two corynebacteria, all organisms were Gram-negative rods varying from long slender forms (length, 5·0μ; width, 0·4μ) to short stout 'coccobacilli' (length, 1·0μ; width, 0·6μ).

Morphology under phase contrast. The Gram-negative bacteria again varied from long slender rods to short stout rods, though there was a marked tendency for longer and more slender rods to develop in liquid media than on solid media.

Motility and flagella staining. Of the 6 motile bacteria, peritrichous flagella were demonstrated for 3 strains and polar flagella (1–2/rod) for 3 (see above).

Colony characteristics. Colony pigmentation of the Gram-negative non-motile bacteria ranged from deep yellow or orange-yellow to pale yellow or yellow-green. The bacteria comprising each of the 4 main groups above had similarly pigmented colonies. The typical appearance of colonies at 5 days was: yellow pigmentation, 1·5–0·5 mm. in diameter, circular, convex, surface smooth and shiny, entire edge, translucent by transmitted light. The cytophaga, NCMB 292, was an agar liquefier.

Appearance in sea-water broth. Almost all isolates grew slowly in sea-water broth, visible turbidity appearing in 2 or 3 days. Nearly all isolates gave a moderate degree of turbidity throughout the medium; the presence or absence of surface growth and deposits were variable characters.

Effect of environmental conditions on growth

Temperature range of growth. Forty-three isolates grew in either the 0° to 30°, or 0° to 25° temperature ranges; only 9 did not grow below 10°. Only strains NCMB 300 and 411, the cytophaga NCMB 11, and the corynebacterium NCMB 280, grow at 37°.

Heat resistance. Thirty-nine isolates, including all members of groups 2, 3 and 4, were not resistant to 45° for 1 hr., and of these, 28 were unable to withstand 45° for 30 min. In fact 13 isolates, mainly members of group 2, were not resistant to 45° for 15 min. Of the remaining 23 organisms only two (NCMB 411; the corynebacterium NCMB 280) were resistant to 55° for 30 min. Of the 58 organisms examined, 36 grew normally after 24 hr. at 37°, this number decreasing to 27 after 48 hr. Not unexpectedly those strains which showed loss of viability after 24 hr. at 37° were least resistant to higher temperatures and those which survived after 48 hr. at 37° were all able to withstand 45° for 1 hr.

Anaerobic growth. Only four organisms, NCMB 263, 268, 279 and the vibrio NCMB 283, grew anaerobically, producing only slight growth.

Salinity range of growth. Fifty-one strains grew in 6% (w/v) NaCl and 6 of these (NCMB 289, 296, 299, 409, the corynebacterium NCMB 299, and the unclassified strain NCMB 297) grew in 10% (w/v) NaCl; with the majority growth was better on the lower concentrations but at 6% NaCl the growth was usually more than scanty. Nine strains did not grow at a concentration greater than 4% (w/v) NaCl and two (NCMB 291, 408) only tolerated 2% (w/v) NaCl. A number of isolates NCMB 266, 279, 285, 289, 290, 293; the cytophagas NCMB 11, 292) showed a marked decrease of growth at the lowest concentration (0·5%, w/v, NaCl).

Sensitivity to antibiotics and a 'vibriostatic' compound. Fifty-seven organisms

were markedly sensitive to chloramphenicol (radius of inhibited growth > 10 mm.), the exceptions (NCMB 262, 273, 411, the vibrio NCMB 283 and the unclassified strain NCMB 297) being sensitive to a lesser degree. Fifty-four isolates were sensitive to streptomycin and 36 to penicillin; the organisms comprising groups 1 and 3 were almost all markedly sensitive to penicillin. Twenty-one isolates were sensitive to the vibriostatic compound, 14 being group 1 strains, but these isolates were non-motile and would not be considered as Vibrio species (*Bergey's Manual*, 1957).

Physiological features

Gelatin liquefaction. Forty-two (67 %) isolates were slow gelatin liquefiers, the reactions ranging from complete liquefaction in 14 days to slight liquefaction in 6 weeks. With one exception the liquefaction was saccate or infundibuliform; the exception, strain NCMB 292, was stratiform. Seventeen strains did not liquefy gelatin and three did not grow under the test conditions (see Table 3). These results confirm those of Gary (1950) who, in a study of 30 non-marine flavobacteria, found that 83 % liquefied gelatin. An analysis of the properties of those Flavobacterium species listed in *Bergey's Manual* (1957), which had been isolated from marine sources ('marine flavobacteria') showed 88 % to be gelatin liquefiers. Ferrari & Zannini (1958), with 46 strains considered by them to be flavobacteria, found that 17 % liquefied gelatin. Several representative cultures received from these authors had a morphology and colony appearance typical of Corynebacterium species (e.g. NCMB 644, 667). It seems almost certain therefore that they were studying a heterogeneous collection of bacteria and for this reason their results will not be discussed further.

Action on litmus milk. Forty-two (67 %) strains produced a variety of changes in litmus milk; in no case were these rapid. The most common action was the reduction of litmus frequently associated with either peptonization or an acid or alkaline reaction; 8 strains produced an alkaline reaction with no other change. With the exception of group 1 strains, which failed to change the medium, groups 2, 3 and 4 produced no characteristic reactions (see Table 3). Of the bacteria examined by Gary (1950) 49 % produced changes in litmus milk and 56 % of the marine flavobacteria in *Bergey's Manual* (1957) are listed as producing changes.

Reduction of nitrate to nitrite. Only 5 isolates (NCMB 280, 283, 286, 296, 300) reduced nitrate to nitrite, strain NCMB 286 continuing the reduction to ammonia or nitrogen gas. It should be noted that only two of these isolates are flavobacteria and these are motile (see Discussion). Gary (1950) found only 13 % of his flavobacteria to be nitrate reducers, whereas 47 % of the marine flavobacteria in *Bergey's Manual* (1957) are listed as nitrate-reducing species.

Ammonia production. Forty-two (67 %) organisms gave a positive ammonia reaction but only 6, including the group 3 isolates, were strongly positive (see Table 3). Of the marine flavobacteria in *Bergey's Manual* (1957), 75 % are given as positive.

Hydrogen sulphide formation. Fifty-two (84 %) of the isolates produced hydrogen sulphide (see Table 3). These results contradict those of Gary (1950), who found that none of his flavobacteria produced hydrogen sulphide, and those of *Bergey's Manual* (1957) which lists 33 % of the marine flavobacteria as positive.

Indole formation. None of the organisms gave an indole-positive reaction. All

Table 3. *Biochemical reactions of the Gram-negative non-motile isolates*

	Fermentations*															Other reactions†					
NCMB no.	Arabinose	Xylose	Rhamnose	Glucose	Fructose	Mannose	Galactose	Sucrose	Maltose	Lactose	Trehalose	Raffinose	Starch	Mannitol	Salicin	Gelatin	Litmus milk	NH_3	H_2S	Starch hydrolysis	Oxidase
244 (Typical group 1 isolate)	–	+	+(*a*)	+	+	+	+(*b*)	+	+(*c*)	–	–	–	–	+(*d*)	+(*e*)	+(*f*)	–(*g*)	–	+	–(*h*)	–
260 (Typical group 2 isolate)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	alk. red. (*i*)	+	+	–	+(*j*)
249 (Typical group 3 isolate)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	red.	+	+	+	–
253 (Typical group 4 isolate)	–(*k*)	–(*l*)	–	+	+(*m*)	+	+	–(*n*)	+	+	+(*o*)	+(*p*)	+(*q*)	–(*r*)	–(*s*)	+(*t*)	red.	+(*u*)	+(*v*)	+(*w*)	–
Cytophagas																					
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	+
292	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	+
Miscellaneous flavobacteria																					
266	–	–	–	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	+	+	–
274	–	–	–	+	–	–	–	–	+	–	–	–	+	+	–	+	red. pep.	+	+	+	+
277	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	+	–	–	–	P	+
278	–	–	–	+	+	+	–	+	+	–	–	–	+	+	–	+	red. pep.	+	–	+	+
279	+	–	–	+	+	+	+	–	+	+	–	+	–	–	–	+	–	+	+	–	–
282	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	ac. red.	–	+	+	–
288	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	+	+	P	–
290	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	NG	–	+	+	P	–
291	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	P	+
293	+	+	–	–	–	–	+	–								NG				P	
408	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	alk.	–	–	–	+
409	+	–	+	+	+	+	+	+	+	–	+	–	–	–	–	+	red.	+	–	P	+
410	+	+	–	+	+	+	+	+	+	–	+	+	–	–	–	–	red.	+	–	P	+
411	–	–	–	+	+	+	–	+	+	–	–	–	–	+	+	+	red. pep.	+	+	P	–

* +, acid produced; –, no acidity in 28 days.

† NG, no growth; alk., alkaline; ac., acid; red., reduced; pep., peptonized; P, partial hydrolysis.

(*a*) No acid produced by NCMB 248 and 273. (*b*) No acid produced by NCMB 245, 252, 259, 261, 263, 273 and 295. (*c*) No acid produced by NCMB 252 and 263. (*d*) No acid produced by NCMB 273. (*e*) No acid produced by NCMB 273. (*f*) No liquefaction by NCMB 248 and 273. (*g*) NCMB 261, 263 and 298 produced acid conditions and NCMB 273 alkaline conditions. (*h*) Partially hydrolysed by NCMB 248. (*i*) No reduction by NCMB 250. (*j*) NCMB 250, 251, 262, 264, 265, 276 and 284 oxidase negative. (*k*) Acid produced by NCMB 256. (*l*) Acid produced by NCMB 255 and 285. (*m*) No acid produced by NCMB 257 and 275. (*n*) Acid produced by NCMB 247, 275 and 285. (*o*) No acid produced by NCMB 256. (*p*) No acid produced by NCMB 256, 257 and 275. (*q*) No acid produced by NCMB 247 and 285. (*r*) Acid produced by NCMB 275. (*s*) Acid produced by NCMB 275. (*t*) No liquefaction by NCMB 256, 257 and 285. (*u*) No ammonia formed by NCMB 247. (*v*) No H_2S formed by NCMB 285. (*w*) No hydrolysis by NCMB 247 and 285.

marine flavobacteria in *Bergey's Manual* (1957) are said to be negative, whereas Gary (1950) found 17% of his flavobacteria to be positive.

Hydrolysis of urea. Only 6 organisms (NCMB 283, 286, 295–7, 300) hydrolysed urea; of these only NCMB 295 is a member of one of the main groups.

Trimethylamine oxide reduction. Only NCMB 286, Pseudomonas species, was found to reduce trimethylamine oxide.

Production of acid from carbohydrates. Thirty-six (58%) isolates produced acid only from one or more carbohydrates. Acid production was not generally noted until after incubation for 1 week and only slight to moderate amounts were then present (see Table 3); of the carbohydrates not listed, inulin was attacked with acid formation by 4 strains (NCMB 247, 266, 297, 410), sorbitol by one strain (NCMB 244), whilst dulcitol and inositol were not attacked by any of the isolates. Glucose was the most commonly attacked carbohydrate though three isolates which dissimilated galactose with acid production failed to attack glucose. Gary (1950) found the formation of acid from glucose, sucrose, maltose, lactose and mannitol varied between 10 and 23%; a few flavobacteria formed acid and gas from glucose and sucrose but Gary considered that the 'fermentative' strains should be excluded from the genus. Little information on carbohydrate dissimilation by the marine flavobacteria is given in *Bergey's Manual* (1957) except that 70% formed acid from glucose.

Glucose utilization. Only 2 strains (NCMB 297, 409) were oxidative and 3 (NCMB 278, 282–3) fermentative. Seventeen isolates, including 13 from group 2, produced an alkaline reaction in the aerobic tube only, and 31 organisms did not change the medium; the remaining 9 isolates did not grow. Thirty-three organisms formed acid from glucose and yet only 5 gave a positive result in Hugh & Leifson's medium; the remaining 28 organisms either did not change the medium or did not grow. With the strains which failed to produce any change the growth was frequently very slight; this may account in part for these unexpected results. Also the strains were relatively late and weak acid producers and the indicator may not have been sufficiently sensitive. Acid production was more marked from fructose than from glucose, and fructose was therefore substituted for glucose in the medium. Acid-producing bacteria were examined but growth was again slight and no pH change was noted. Thus it appeared that these anomalous results were not due to the insensitivity of the indicator but were possibly due to the indicator exerting an inhibitory effect. To confirm this Andrade's indicator was used as an alternative. Though less sensitive than bromthymol blue a definite acid reaction was obtained with a number of organisms which previously had shown no change. However a few strains did not grow and others showed only slight growth with no pH change and anomalous results remained.

Production of 2-ketogluconic acid. Only 3 isolates grew in the gluconate medium and of these, NCMB 296 and 300 (both motile flavobacteria; see Discussion) gave a positive reaction. No flavobacteria have been reported to produce 2-ketogluconic acid.

Starch hydrolysis. Thirteen (21%) isolates hydrolysed starch and of these, 9 continued the breakdown to acid; also, 11 other strains partially hydrolysed starch (see Table 3). Gary (1950) found that 40% of his flavobacteria hydrolysed starch.

Tributyrin hydrolysis. Only 18 isolates grew on the test medium and 15 of these

hydrolysed tributyrin, including 6 isolates from group 1, one from group 3, the cytophaga NCMB 11, and NCMB 408, 409 and 411; the remainder consisted of the motile strains, NCMB 296 and 300 and the corynebacteria.

Growth in Koser's citrate medium. Only the Corynebaterium species NCMB 299, the unidentified species NCMB 297, and the motile flavobacterium NCMB 296, utilized citrate; Gary (1950) found that 43% of his flavobacteria grew in citrate.

Voges–Proskauer and methyl red tests. None of the strains was Voges–Proskauer-positive and only 2 (the vibrio and the unidentified species) were methyl red-positive; 12 isolates did not grow.

Digestion of chitin. Of the 29 isolates which grew in the medium containing chitin none digested this substrate.

Decomposition of cellulose. All isolates grew on the test medium but none appeared to decompose the cellulose.

Blood agar. Only 32 isolates grew on blood agar; none haemolysed the blood.

Actomyosin agar. All the isolates grew on this medium but none attacked the actomyosin.

Catalase and oxidase formation. All isolates were catalase-positive and 21 were oxidase-positive (see Table 3).

Table 4. *Nutritional requirements*

Medium	No. strains first showing growth	NCMB number
Basal medium 1	2	296, 300
Amino acid medium 2	10	249, 254, 269, 280, 286, 287, 294, 11, 409, 411
Growth factor medium 3	6	273, 275, 281, 284, 292, 297
Growth factor medium 4	18	247, 250, 251, 253, 256, 257, 262, 264, 265, 267, 270, 271, 272, 276, 278, 282, 299, 408
Purine + pyrimidine medium 5	3	260, 268, 274
Peptone medium 6*	20	244, 245, 246, 248, 252, 255, 258, 259, 261, 263, 266, 279, 288, 289, 290, 291, 293, 295, 298, 410

* Strain NCMB 285 only grew on the addition of sea water to this medium.
Strains NCMB 277 and 283 failed to grow in any of the test media.

Nutrition

The nutritional requirements of the isolates are given in Table 4, which shows the generally complex requirements of the majority of isolates tested. Only the motile flavobacteria (NCMB 296, 300) grew in the basal medium. The 10 isolates which grew in the amino acid medium included all group 3 strains, the two Pseudomonas

species, one each from groups 1 and 2, and the cytophaga NCMB 11; those which grew in the growth factor medium no. 3 included the cytophaga NCMB 292, two group 1 organisms, one each from groups 2 and 4 and the unidentified species NCMB 297. Of the 18 isolates which grew in the growth factor medium no. 4, 10 were group 2 strains and 4 group 4 strains. Of the 20 isolates which required peptone, 12 were group 1 organisms. The isolate NCMB 285 which only grew on the addition of sea water also failed to grow in distilled water nutrient broth and it can only be assumed that it had complex ionic requirements. The two organisms which did not grow in any test media grew in sea-water peptone water; this anomalous result may have been due to antagonism between nutrients at the concentrations supplied.

The results are generally in agreement with the few publications on this topic. No previous paper has dealt specifically with the nutrition of marine flavobacteria. It is apparent from two contributions on non-marine flavobacteria that they have complex nutritional requirements (Prince, Beck, Cleverdon & Kulp, 1954; Prince & Cleverdon, 1955). In a study of the nutrition and metabolism of marine bacteria, MacLeod, Onofrey & Norris (1954) found the flavobacteria to have diverse requirements: one strain required unknown growth factors; three required amino acid mixtures; one strain had simple requirements. Weeks & Beck (1960) found *Flavobacterium aquatile* to require only thiamine as a growth factor, though an enzymic hydrolysate of casein, glucose and inorganic salts were also supplied.

Swarming

The cytophagas NCMB 11 and 292, all 6 group 4 isolates and 12 of the 14 group 1 isolates all produced spreading colonies in one or more of the media used. A clear gradation was seen in the colonies developed by the strains which showed swarming tendencies. When grown on high peptone concentrations the colonies, even of the Cytophaga species, were discrete and corresponded to those formed by typical Flavobacterium species. A tendency for colonies to spread became more evident with decreasing peptone concentrations; strains frequently produced 'intermediate' colonies which had raised centres and flattened peripheries but did not exhibit true swarming. In the lowest peptone concentration swarming was less in evidence, the colonies being minute because of the extreme lack of available nutrients.

Normal sea-water agar contained 1·0 % (w/v) peptone with 1·5 % (w/v) agar and on this medium 4 isolates developed spreading colonies after incubation for 10 days; with 2·0 % (w/v) agar this number was increased to 6 isolates. When the peptone concentration was lowered to 0·25 % (w/v) swarming was observed with 15 isolates and a further 3 isolates gave rise to 'intermediate' colonies. These 18 isolates swarmed readily at the lower peptone concentrations. Two isolates only developed spreading colonies on 0·01 % (w/v) peptone. By increasing the peptone concentration to 4 % (w/v) only the two cytophagas NCMB 11 and 292 swarmed; at 8 % (w/v) peptone, swarming was completely inhibited. Isolates which failed to form spreading colonies on any of the media showed only a progressive diminution in colony size with lower peptone concentrations. *Flavobacterium aquatile* did not swarm on any of the test media.

When the swarming edges of colonies of the cytophagas NCMB 11 and 292 were observed microscopically, gliding motility was seen distinctly only on 1 and 0·25 % (w/v) peptone agar; gliding motility was not seen with colonies of these organisms

Table 5. *Cell-wall composition of representative strains*

NCMB number	Amino acids												Sugars			
	Alanine	Arginine	Aspartic acid	Di-amino-pimelic acid	Glut-amic acid	Glycine	Leucine	Lysine	Proline	Serine	Tyro-sine	Valine	Galac-tose	Glucose	Man-nose	Rham-nose
244	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−
249	+	−	+	+	+	+	+	+	−	+	−	+	−	−	−	−
251	++	+	++	++	++	++	++	+	+	++	+	++	++	++	++	++
253	++	+	++	++	++	++	++	+	+	++	+	++	−	+	+	+
F. aquatile	++	−	++	++	++	++	++	−	−	++	+	++	++	++	++	−
11	++	−	++	++	++	++	++	+	+	++	−	++	−	+	−	−

++, Moderate or intense spot; +, weak spot; −, not detected.

on 4 and 8% (w/v) peptone agar. A gradation in degree of motility corresponding to the colony form was thus suggested, confirming that gliding motility was characterized macroscopically by spreading growth of the colony. Distinct gliding motility was not observed microscopically with any other isolates examined but this may be explained by the relatively restricted degree of spread produced, which was roughly equal to that of the cytophagas NCMB 11 and 292 on 4% (w/v) peptone agar.

Examination of cell walls

Staining of cell walls. Though *Bacillus cereus* when used as a readily stainable control gave excellent results, all the test isolates were less satisfactory. This was not unexpected since Hale & Bisset (1956) found that Gram-positive bacteria were far more readily stainable than Gram-negative ones. An additional disadvantage was that, in the majority of cases, the cells were so small or slender that even with a good staining technique the walls would be difficult to discern. However a few definite results were obtained which could be summarized as follows: (1) cell walls were not seen in the case of the cytophagas (NCMB 292, 11); (2) distinct cell walls were observed in groups 2 and 3 isolates and also in some of the 14 Gram-negative non-motile isolates which were not grouped; (3) the remaining isolates included those in which cell walls could probably be discerned (e.g. other isolates in groups 2 and 3) and those where the demonstration of walls was virtually impossible because of the small size of the cells (e.g. group 1 isolates).

Cell-wall analysis. The results (see Table 5) show that the 6 isolates examined formed a homogeneous group with regard to the amino acids identified. Those amino acids which gave the stronger spots with strains NCMB 251 and 253, the cytophaga NCMB 11 and *Flavobacterium aquatile* were the only ones detected in strains NCMB 244 and 249, where smaller amounts of material were available (see Methods). The isolates studied had a fairly wide range of amino acids in their cell walls; this is true of most Gram-negative bacteria (Salton, 1956). The results for the cytophaga confirm those of Mason & Powelson (1958) who, by hydrolysis of the cell wall of *Myxococcus xanthus*, identified 17 amino acids including the 12 detected in the present work; these authors concluded that the cell walls were similar to those of other Gram-negative bacteria. A quantitative difference was observed with the sugars, the spots being substantially stronger with strain NCMB 251 and *F. aquatile* than with strain NCMB 253 and the cytophaga NCMB 11. The absence of sugar spots with strains NCMB 244 and 249 may probably be explained by the smaller amounts of material chromatographed. The detection of sugars in the cell wall of the cytophaga confirms the findings of Mason & Powelson (1958) who identified galactose, glucose and rhamnose from *M. xanthus*. The failure to detect glucosamine and muramic acid was probably due to the low concentrations of hexosamines normally found in Gram-negative bacteria (Salton, 1956), though the isolation of an unidentified hexosamine from *M. xanthus* (Mason & Powelson, 1958) suggests the concentrations may differ.

DISCUSSION

It is probable that each of the groups 1–4 merits species rank and the members of the groups are strains. Groups 2 and 3 are sufficiently close to the description of the genus *Flavobacterium* in *Bergey's Manual* (1957) to justify their inclusion

in the genus, although a comparison between these groups and the species listed in *Bergey's Manual* shows that neither of the groups is sufficiently like any named species as to be identical to it; in addition to groups 2 and 3, the 14 Gram-negative non-motile isolates are also regarded as Flavobacterium species. Groups 1 and 4 would also be included as Flavobacterium species except that spreading colonies were formed. The inclusion of the motile bacteria (NCMB 296, 300) in the genus is questionable since their properties are so very different from the non-motile flavobacteria examined. Since polarly flagellate yellow-pigmented organisms have been excluded from the genus the retention of peritrichous forms could be further criticized; a new genus could therefore be suggested for the peritrichous forms.

The absence of a demonstrable cell wall is given as a characteristic of myxobacteria in *Bergey's Manual* (1957) but the chemical composition of the cell walls of the cytophaga examined clearly has certain affinities with the cell walls of the flavobacteria. An additional property of myxobacteria is reputed to be that of flexion or vigorous bending of individual organisms; this has been observed by many workers (e.g. Bachmann, 1955). However no flexion was noticed in the present work and it may be a feature of doubtful significance as suggested by Anderson & Ordal (1961).

The only property which distinguishes flavobacteria from cytophaga experimentally appears to be that of spreading growth. Any non-flagellate bacterium which shows spreading growth should exhibit some form of gliding motility. Gliding motility is accepted as one of the principal distinguishing properties of the genus *Cytophaga* (*Bergey's Manual*, 1957); thus all non-flagellate bacteria which have this property may be considered as myxobacteria. The majority of group 1 and all group 4 isolates swarmed readily under certain conditions; they are therefore identified as presumptive Cytophaga species. Those isolates from group 1 which did not swarm are retained in the group because of their otherwise similar properties. Therefore, besides the cytophagas NCMB 11 and 292, a further 20 isolates are likely to be cytophagas.

There is a great similarity between the properties of the swarming and non-swarming isolates in this work; the swarming isolates have a greater heat resistance than the typical flavobacteria and this is the only other characteristic which differentiates these two groups. The similarity between the swarming and non-swarming isolates examined suggests that the affinities between flavobacteria and cytophagas may be stronger than appears. Further work is necessary to establish whether or not the properties of swarming, flexion and non-stainable cell walls are specific to myxobacteria; the study of this problem must be extended to include both non-marine and marine named species of the two genera.

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Cellular Morphology of Form 2 Mycobacteria in Slide Culture

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SUMMARY

Form 2 of a strain of *Mycobacterium tuberculosis* var. *hominis* was isolated. The cellular morphology of this organism when growing in glycerol agar slide culture is described. The form 2 strain grew by the initial production of septate filaments which soon ramified as a result of pseudobranching. The filaments fragmented early into bacillary elements and much later into coccoid elements. Endospores were formed within some of the bacillary elements. The young cells were Gram negative and the older cells Gram positive. The cells were never acid-fast. Growth occurred in aerobic and anaerobic culture, but the morphological changes progressed more rapidly under anaerobic conditions. The strain has many characteristics also found in some members of the Actinomycetaceae; however there are also differences, the most important of which is endospore formation. Thus the strain cannot yet be classified.

INTRODUCTION

In a previous publication (Csillag, 1961), it was reported that rapidly growing organisms, which were not acid-fast, were obtained from cultures of mycobacteria (the majority of the strains were *Mycobacterium tuberculosis*) grown under special conditions, when these cultures were inoculated on plates of nutrient agar. The rapidly growing organisms, at the time of their isolation, were described as Gram-negative spore-bearing rods or as Gram-negative cocci often arranged in chains. It was therefore suggested that mycobacteria might be dimorphic in the same sense that some of the human pathogenic fungi are known to be dimorphic. The acid-fast mycobacteria, as usually described, were termed 'form 1' and the organisms obtained on nutrient agar were termed 'form 2'. Subsequently, form 2 organisms have been isolated from all of the 123 strains of *M. tuberculosis* examined. The form 2 organisms were similar in their main characteristics, though variations between strains occurred. The life cycle of one of the strains, selected at random, has now been studied in detail. Reported here is a description of the cell morphology of this strain when grown under undisturbed conditions in slide culture. This particular aspect of a complex life cycle was chosen, in part, because it may throw light on the taxonomic position of the strain, and in part because slide culture is an easy means of identifying form 2 organisms and of distinguishing them from contaminants.

METHODS

Strain. The strain (I 1413) of *Mycobacterium tuberculosis* used was isolated from the sputum of a British patient with pulmonary tuberculosis. It was virulent in the guinea-pig, sensitive to isoniazid, streptomycin and *p*-aminosalicylic acid and produced nicotinic acid (Runyon, Selin & Harris, 1959). The strain was maintained on Löwenstein–Jensen medium at 37° for 5 months before the isolation of form 2 organisms was begun. (The strain is deposited in the National Collection of Type Cultures, Colindale Avenue, London, N.W. 9 as NCTC 10.280.)

Media. (1) The Löwenstein–Jensen medium used was without potato starch (Jensen, 1955); (2) nutrient broth was prepared from meat extract + peptone (Oxoid, No. 2, Oxo Ltd., London); (3) nutrient agar was made by adding 1·4 % (w/v) agar to the nutrient broth; (4) glycerol agar was prepared by adding 7 % analytical grade glycerol to nutrient agar; (5) 7H-10 oleic acid–albumin agar (Cohn, Middlebrook & Russell, 1959). All media were incubated at 37° for 2–3 days before use, as a test of their sterility.

Isolation and maintenance of form 2 organisms. After preliminary purification of the form 1 strain on 7H-10 medium plates, form 2 organisms were obtained, as previously described (Csillag, 1961), by subcultivation from growth on aerated Löwenstein–Jensen medium slopes to nutrient agar plates. The form 2 strain was purified twice by single colony selection from nutrient agar plates which had been incubated for 3 days at 37°. Nutrient agar slopes in screw-capped bottles were inoculated from colonies on the second plate. The slopes were incubated with loose caps at 37° for 3 days until sporulation had occurred; the caps were then closed and the cultures maintained at room temperature. The strain was re-purified and sporulation allowed to occur every two months. At the start of the experiments to be described, the strain had been maintained for 12 months and the last subculture was one week old (the initial culture).

Preparation of slide cultures. A 1·2 × 4 in., flat-bottomed specimen bottle, plugged with cotton-wool and containing a 3 × 1 in. microscopic slide and 10 ml. glycerol agar, was autoclaved. The bottle was then allowed to cool in a sloped position, so that the slide was at right angles to the surface of the medium. All the slide cultures were prepared at the same time and from the same batch of medium. A standard inoculum, containing vegetative forms only was prepared for the slide cultures in the following manner. The initial culture was plated out on nutrient agar. After incubation at 37° for 24 hr., a suspension was prepared by adding a single colony to a $\frac{1}{4}$ oz. screw-capped bottle containing 0·4 ml. sterile distilled water and glass beads; the bottle was then shaken by hand for a few seconds. The suspension consisted of Gram-negative rods, some containing endospores (Pl. 1, fig. 1). A loopful of the suspension was inoculated into a $\frac{1}{2}$ oz. screw-capped bottle containing 8 ml. nutrient broth which was shaken mechanically at 37° for 6 hr. The culture was composed of Gram-variable, vegetative rods; neither endospores nor free spores were present (Pl. 1, fig. 2). A loopful of this culture was then inoculated on to the slide cultures along the line where the slide entered the medium, thus allowing the organism to grow first on to a thin layer of medium on the slide and then on to the glass surface of the slide. The plugs of the slide cultures incubated under aerobic conditions were covered with aluminium foil. The purity of the inoculum was

checked by plating on nutrient agar, and uninoculated slide cultures were incubated as medium controls.

Investigation of slide cultures. Several slide cultures were incubated at 37° either aerobically or anaerobically in a McIntosh & Fildes jar, controlled with a Lucas semi-solid indicator. At intervals one of each of the two types of culture was examined and then discarded. The slide was removed with forceps, allowed to dry under an ultraviolet lamp and fixed with methanol for 5 min. The growth on the slide close to the bottom of the specimen tubes, where condensation water was usually present during incubation, was examined. Preparations were stained by the Gram and Ziehl–Neelsen methods as described by Csillag (1961). Staining with malachite green (1 % malachite green in 1 % aqueous phenol, filtered before use) was done for 5 min. Cell walls were stained by the tannic acid–crystal violet method (Bisset, 1955).

RESULTS

Aerobic incubation

Morphology under low-power magnification. In slide cultures incubated under aerobic conditions, the following changes occurred in the cell morphology of the form 2 organisms, as observed under a magnification of × 120. At one day, long, narrow filaments were seen growing away from the junction of the slide and the medium. The filaments had short side branches (Pl. 1, fig. 3). At about 7 days, the primary filaments were considerably thicker, the side branches had elongated and further ramification had occurred (Pl. 1, fig. 4). For at least the next 5 weeks, the filaments continued to ramify over further areas of the slide. The younger filaments were narrow but increased in thickness with age. The filamentous arrangement was maintained until the completion of the experiment at 6 weeks.

Morphology under high-power magnification. Examination of the slide cultures under high-power magnification (× 960) yielded further information on the detail of cell morphology and on the mode of branching. The growing ends of the filaments were composed of multicellular strands, about 0·8 μ diameter, in which the cross-walls were only visible when stained with tannic acid–crystal violet (Pl. 1, fig. 5) or when the preparation was examined unstained by phase contrast. In Gram-stained preparations Gram-negative segments alternated with unstained zones, so that the continuity of the filaments was not evident (Pl. 1, fig. 7, left-hand portion). Older portions of the filaments were composed of separate rods lying end to end but without continuity from cell to cell when observed in preparations stained by all four methods. This arrangement suggested that the cells originated by fragmentation of the younger multicellular filaments. The youngest branches were composed of a single strand of cells, but gradually bundles containing parallel strands appeared (Pl. 1, figs. 6, 7). Some of the strands lay so close together that it was difficult to distinguish individual cells (Pl. 1, fig. 6). The majority of the cells in younger branches were still Gram negative but occasionally contained Gram-positive granules (Pl. 1, fig. 6). In older strands an increasing proportion of the cells were uniformly Gram positive (Pl. 1, fig. 7). Gram-positive material could not be distinguished by Ziehl–Neelsen or malachite green staining, the cells stained evenly with malachite green and none of them was acid-fast. At from 7 to 17 days endospores appeared in some cells and free-lying spores were also seen (Pl. 1,

fig. 8). In Gram stains the wall of the endospores was Gram-labile and the interior unstained; in Ziehl–Neelsen stains the wall was blue and the interior unstained, and the spores stained uniformly with malachite green.

The branching observed under low-power magnification proved to be pseudo-branching when examined under the higher power. A cell lying in the outer strand of a bundle changed its direction of growth and produced a filament at an angle to the main bundle (Pl. 1, fig. 7). Further strands then grew, probably from the main bundle, to lie parallel to this new filament.

After about 6 weeks some of the cells lying in strands were seen to fragment into coccoid elements. This process occurred in only a few cells, particularly those in the lower part of the slide culture near the water of condensation. At first the cells containing the coccoid elements were faintly stained, but later they disappeared, leaving the coccoid bodies lying in chains, in pairs or singly (Pl. 1, fig. 9). While intracellular, the coccoid bodies varied in shape (spherical or oval), but they were always spherical when extracellular. In both situations they ranged from 0·5–1·0 μ in diameter. Both the coccoid bodies and the cells were Gram positive, not acid-fast and stained by malachite green, but the coccoid bodies were stained more intensely.

Smear preparations. Smears taken from the junction of the slide and the agar surface invariably failed to show any filamentous arrangement, thus suggesting that the filaments were easily disintegrated. Predominantly Gram-negative rods were found in smears from young cultures and, with increasing age, there were also Gram-variable and Gram-positive rods present. Smears from the oldest cultures were composed of Gram-positive rods (some containing endospores), free lying spores and cocci, giving the impression of a mixture of different bacteria.

Anaerobic incubation

The sequence of changes described in slide cultures grown aerobically also occurred in slide cultures incubated anaerobically, though they took place more rapidly. In anaerobic culture the changes may be summarized as follows: (1) ramification occurred at about 1 day instead of at about 7 days (Pl. 1, fig. 10). (2) The change from Gram-negative to Gram-positive cells appeared earlier. (3) Fragmentation of the filaments into individual cells occurred earlier in younger cells. Furthermore, the majority of the cells then lost their filamentous arrangement and were arranged randomly on the slide. (4) Fragmentation into coccoid elements was seen within 14 days (Pl. 1, fig. 11); although occurring infrequently, it was found in a higher proportion of the cells than in aerobic culture. (5) The free coccoid elements continued to divide by binary fission to form diplococci, tetrads and sarcina-like configurations, in which the individual cocci varied considerably in size (Pl. 1, fig.12). This division of the liberated coccoid elements was never seen in aerobic cultures. (6) In making smears it was apparent that the majority of the growth occurred within and not on the surface of the glycerol-agar medium of the slide-cultures.

DISCUSSION

The main characteristics of the organism described are the formation of a pseudobranching filament which rapidly fragments into bacillary elements, followed much later by fragmentation into coccoid elements. Similar characteristics are known to be the main features of some organisms classified in the Order Actinomycetales. Waksman & Henrici (1943) included in this order three families: (1) Mycobacteriaceae; (2) Actinomycetaceae; (3) Streptomycetaceae. The family Actinomycetaceae is subdivided into two genera: (1) *Actinomyces* (anaerobic, microaerophilic); (2) *Nocardia* (aerobic). Members of the family Actinomycetaceae (Buchanan) are defined as organisms forming a vegetative branching mycelium which fragments into bacillary and coccoid elements (*Henrici's Molds*, 1947; *Bergey's Manual*, 1948; Waksman, 1959). The fragments have been termed oidiospores, arthrospores, segmentation spores or fragmentation spores by different authors; their shape and size is similar to rod-shaped and spherical bacteria (Waksman, 1959). These fragments continue to divide by transverse fission and, when smears are made, the arrangement of the mycelial fragments is disturbed and the smears appear to consist of ordinary, mixed bacteria (*Henrici's Molds*, 1947). When subcultured into a suitable fresh medium, the fragments germinate to form a new mycelium (Waksman, 1959). In some strains parallel bundles of filaments have been found (Baldacci, Gilardi & Amici, 1956); Gram-positive granules have been seen in the Gram-negative cytoplasm of the cells of some species (*Henrici's Molds*, 1947).

Although there are many similarities between the characteristics of the strain described in the present paper and the characteristics of the Actinomycetaceae, certain differences are apparent. However, when account is taken of the more recent publications on the morphology of the Actinomycetaceae the differences are less substantial and can be summarized as follows. (1) True branching was not observed in the filaments of the strain. Bisset & Moore (1949) showed that true branching, which occurs in the Streptomycetaceae, is not seen in the Actinomycetaceae and Bisset (1955) suggested that members of the Actinomycetaceae should not be regarded as forming true mycelia, but as being composed of a mass of separate, filamentous bacteria. (2) The present strain had cross-walls even in the youngest filaments, whereas the young substrate mycelium of the Actinomycetaceae is generally considered to be non-septate initially (Ørskov, 1923; *Henrici's Molds*, 1947). However, Bisset & Moore (1949) showed, by using the tannic acid–crystal violet stain, that the filaments were really composed of numerous short cells. (3) The youngest cells of the present strain were Gram negative. Although the Actinomycetaceae are usually considered to be Gram positive, occasional Gram-negative strains have been reported (Waksman, 1959). (4) The method of formation of endospores by the present strain (Pl. 1, fig. 8) appears to differ from the description of spore formation in the Actinomycetaceae. (5) Fragmentation into coccoid elements occurred at about 42 days in aerobic culture and at about 14 days in anaerobic culture. The formation of coccoid fragmentation-spores in the Actinomycetaceae is usually complete in 1–3 days (Krassilnikov, 1959; Erikson, 1949). On the basis of the majority of the characteristics the present strain should be classified in the family Actinomycetaceae. However, the production of structures which morphologically resemble endospores appears to exclude this possibility. Endo-

spore production suggests classification among the Bacillaceae, but the continued multiplication of the coccoid elements by binary fission renders this classification untenable. It will be shown in a later publication that all form 2 mycobacteria, as well as the present strain, produce coccal bodies under microaerophilic conditions and that these continue to multiply regularly. Only certain aspects of the morphology of a typical form 2 strain are described in the present publication. The final classification of the strain must depend on a fuller account of the life cycle.

There have been several claims that rapidly growing, not acid-fast, saphrophytic and extremely pleomorphic organisms, resembling species of the Actinomycetaceae, have been obtained from various strains of mycobacteria (Coppen-Jones, 1895; Dostal, 1910; Karwacki, 1930; Møllgaard, 1931; Vaudremer, 1931). Weissfeiler (1933), from his own experience and by reviewing the literature, supported the theory of earlier workers that the genus *Mycobacterium* is a true member of the Actinomycetaceae; it is not acid-fast in its saprophytic form and appears only in the form of an acid-fast rod when adapted to parasitic life. Weissfeiler claimed that the parasitic form reverted to the saprophytic form under the influence of factors such as the old age of the cultures, frequent subcultivation, media poor in nutriments and the presence of toxic agents. According to these views, the acid-fast form of *Mycobacterium tuberculosis* might be only a single stage in a more complex life cycle.

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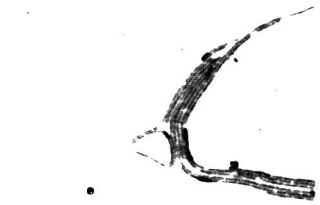
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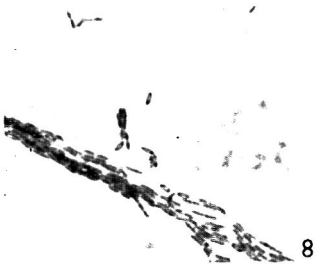
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EXPLANATION OF PLATE 1

Figs. 1–12. Various stages in the development of a strain of *Mycobacterium tuberculosis*, form 2.

Fig. 1. Nutrient agar plate, 1 day, Gram stain, ×800. Gram-negative rods, with and without endospores.

Fig. 2. Shaken nutrient broth culture 6 hr., Gram stain, ×800. Gram-labile vegetative rods.

Figs. 3–9. Glycerol agar slide cultures, grown under aerobic conditions.

Fig. 3. 1 day, unstained, ×100. Long filaments with short side branches.

Fig. 4. 7 days, Gram stain, ×100. Elongated side branches with further ramification; filaments thicker with increasing age.

Fig. 5. 4 days, cell-wall stain, ×800. Growing ends composed of a single multicellular filament.

Fig. 6. 4 days, Gram stain, ×800. Very tight parallel bundles of strands; many of the Gram-negative cells contain Gram-positive granules; fragmentation into bacillary elements.

Fig. 7. 4 days, Gram stain, ×800. Increasing proportion of the cells stain uniformly Gram positive: development of pseudobranching.

Fig. 8. 17 days, Gram stain, ×800. Intracellular and free endospores.

Fig. 9. 42 days, Gram stain, ×800. Fragmentation into coccoid elements, lying in chains, in pairs or singly.

Figs. 10–12. Glycerol agar slide cultures, grown under anaerobic conditions.

Fig. 10. 1 day, Gram stain, ×100. Extensive ramification.

Fig. 11. 14 days, Gram stain, ×800. Fragmentation into coccoid elements.

Fig. 12. 20 days, Gram stain, ×800. Free coccoid elements dividing by binary fission to form diplococci, tetrads and sarcina-like configurations; sizes of coccoid elements very variable.

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Mutants with Impaired Respiration in *Staphylococcus afermentans*

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SUMMARY

Mutants with small colonies and impaired respiration were induced by 5-fluorouracil in cultures of *Staphylococcus afermentans*, NCTC 7503. The consumption of oxygen in colourless mutants attained 66–77 %, and in mutants with orange colonies 68–77 % of normal values. Inhibitors of protein synthesis (chloramphenicol, tetracycline, chlortetracycline), inhibitors of cell-wall synthesis (penicillin G, phenoxymethylpenicillin), as well as an inhibitor of RNA formation (actinomycin C) affected the growth of the parent and mutant staphylococci to the same extent. On the other hand, inhibitors which affect DNA—namely, degranol, myleran-mannitol and mitomycin C—selectively inhibited the growth of mutant staphylococci with impaired respiration. Mitomycin C induced irreversible depolymerization of DNA in bacterial cells; colourless mutants were 2·7–3·5 times, and mutants with orange colonies 33–45 times more vulnerable to its action than the parent culture. The extraction of DNA from bacterial cells showed that the capacity of DNA for extraction from a complex with proteins was decreased in colourless mutants by 2·8–2·9 times, and in mutants with orange colonies by 5·0–7·3 times.

INTRODUCTION

Mutants with impaired respiration in *Staphylococcus aureus*, induced by ultraviolet radiation (Gause, Kochetkova & Vladimirova, 1957), have been studied in some detail (Gause, Kochetkova & Vladimirova, 1961). These mutants were selectively inhibited by substances which affected the nucleic acids (Gause & Kochetkova, 1962). In this connexion the induction of mutants with 'small colonies' and impaired respiration in some other species of micrococci, and a comparative investigation of their characters, are highly desirable. Some new results along these lines obtained in the work with *Staphylococcus afermentans* are described here.

METHODS

Organisms. The original culture of *Staphylococcus afermentans* was obtained from the National Collection of Type Cultures—NCTC 7503. It was isolated during the course of a routine examination of an eye swab from an 11-year-old boy in the Public Health Laboratory at Wakefield, Yorkshire, in 1948.

Measurement of bacteriostatic action of chemical agents. The methods used, as well as composition of the nutrient media, were described earlier in this Journal (Gause & Kochetkova, 1962).

Manometry. Manometric experiments were carried out in a conventional Warburg apparatus under aerobic conditions at 37°. The organisms were grown on

nutrient agar in Petri dishes for 24 hr. at 37°, washed twice on a centrifuge with 0·8 % NaCl, and afterwards suspended in 0·8 % NaCl in the concentration of about 5 % w/v (wet weight). The dry weight of bacterial suspensions was also estimated, and used in the calculation of oxygen consumption. Suspensions of bacteria immediately before experiments were diluted ten times with M/15 phosphate buffer ($Na_2HPO_4.2H_2O + KH_2PO_4.2H_2O$, pH 7·4) containing 0·5 % of glucose.

Estimation of nucleic acids. Bacteria contained in 100 ml. of medium were washed by centrifugation, and ribonucleic acid (RNA) was extracted from sedimented cells by 1·5 ml. 10 % perchloric acid in the cold (−4°) for 17 hr. The cells were again washed on the centrifuge with 0·5 ml. fresh cold 10 % perchloric acid. The extracts were combined and diluted by an equal volume of 10 % perchloric acid. These extracts did not contain deoxyribonucleic acid (DNA). RNA contents of extracts were estimated by the orcinol test as follows: to 1 ml. of the diluted extract were added 2·5 ml. 0·02 % $FeCl_3$ (in concentrated HCl) and 0·15 ml. 10 % orcinol (in 95 % EtOH). After the tube contents were mixed, the tube was heated for 15 min. in a boiling water bath, cooled, and read photometrically at 660 mμ.

After removal of RNA the bacterial cells were extracted with a fresh 1·5 ml. portion of 10 % perchloric acid at 90° for 15 min., and the contents of DNA in the extract were assayed by the diphenylamine method as follows: to 1 ml. of extract in a small tube were added 2·5 ml. of the diphenylamine reagent (1·0 g. diphenylamine dissolved in 2·0 ml. conc. H_2SO_4 and 98 ml. glacial acetic acid), and the tube was placed in a boiling water bath for 10 min. It was cooled and read photometrically at 540 mμ.

Extraction of DNA by Schmidt & Thannhauser procedure. The method used was that described by Spirin, Belozersky, Shugaeva & Vanushin (1957) as a modification of the original procedure (Schmidt & Thannhauser, 1945). In our experiments 500 mg. of dry bacteria (previously washed by alcohol and ether until washings were colourless) were suspended in 4 ml. 0·5 N-NaOH, and hydrolysed at 37° for 18 hr. The suspension was then acidified in the cold by 70 % $HClO_4$ to make the total concentration of perchloric acid in the solution equal to 1–2 %, and then centrifuged in the cold. The solution containing RNA was discarded, and the sediment containing DNA was washed twice with cold 0·2 N-$HClO_4$, once with alcohol and once with alcohol and ether (1 + 1) in the cold, and finally again with the cold 0·2 N-$HClO_4$ to remove alcohol and ether. A volume of 10 ml. was used for washing at each stage. The wet sediment was neutralized with a saturated solution of $NaHCO_3$, and then extracted by alkaline (pH 8) 10 % NaCl on the boiling water bath for 1 hr. The concentration of DNA in the extract was estimated spectrophotometrically by the difference of optical densities at 595 and 650 mμ. Spectrophotometer SF-4 (U.S.S.R.) was used in our work.

RESULTS

Induction of mutants with impaired respiration. Various mutagenic factors were tried, but positive results were observed only in experiments with 5-fluorouracil. The parent culture of *Staphylococcus afermentans* 7503 was grown at 37° in test tubes in nutrient broth containing 60–70 μg. 5-fluorouracil/ml., and samples from the tubes were daily streaked in various dilutions on plates of nutrient agar. After 4–5 days of incubation of the culture in broth, the subcultures on plates showed

the appearance of small colonies of mutants, dispersed among the colonies of normal bacteria. Some of the mutants were growing in the form of sectors in the colonies of normal organisms. Numerous mutants with small colonies were isolated in the course of this work, and classified into two groups: colourless colonies and colonies of orange colour in various shades. Of the former group, mutants 19 and 44 and of the second group mutants 42 and 22 were used in this study.

Table 1 shows that the consumption of oxygen in colourless mutants attained 66–77 %, and in mutants with orange colonies 68–77 % of normal values, i.e. respiration was impaired to the same degree in these mutants.

Table 1. *Oxygen consumption by washed cell suspensions of parent and mutant cultures of* Staphylococcus afermentans *in the presence of glucose*

	No. of experiments	Q_{O_2}*
Parent culture	12	82·5
Mutant 42 (orange colonies)	7	63·3
Mutant 22 (orange colonies)	5	56·2
Mutant 19 (colourless colonies)	5	54·5
Mutant 44 (colourless colonies)	5	63·9

* The consumption of oxygen in mm^3/hr. calculated for 1 mg. dry weight of bacteria.

Effects of inhibitors. Measurement of minimum inhibitory concentrations of various compounds in nutrient broth showed that inhibitors of protein synthesis in the bacterial cell affected the growth of the parent and mutant organisms to the same degree (0·4–0·5 μg. chloramphenicol/ml.; 0·2–0·3 μg. tetracycline/ml.; 0·4–0·5 μg. chlortetracycline/ml.). Mutant and parent cultures were affected also to the same degree by inhibitors of cell-wall synthesis (0·03–0·05 unit penicillin G/ml.; 0·02–0·03 unit phenoxymethylpenicillin/ml.), as well as by 5-fluorouracil. Addition of thymine (100 μg./ml.) or uracil (100 μg./ml.) produced no effect upon the inhibitory action of fluorouracil.

We were particularly interested in the analysis of the inhibitory action of actinomycin C and mitomycin C, as far as these compounds selectively affect the formation of nucleic acids in the bacterial cell. Experiments showed that mitomycin C selectively inhibited formation of DNA, and actinomycin C selectively affected synthesis of RNA in the logarithmic phase of growth of *Staphylococcus afermentans* (Table 2).

It is of interest that actinomycin C inhibited the growth of parent and mutant cultures of *Staphylococcus afermentans* to the same degree; the minimal inhibitory concentration in broth attained 0·003–0·004 μg./ml. The estimations were made by the tube dilution method, as described by Gause & Kochetkova (1962). In distinction from this, the substances selectively affecting DNA formation—namely, mitomycin C, degranol (1,6-*bis*-(β-chloroethylamino)-1,6-deoxy-*d*-mannitol), and myleran mannitol (1,6-dimethanesulphonyl-*d*-mannitol) also selectively inhibited the growth of mutants with the impaired respiration (Table 3).

The action of mitomycin C is of particular interest, since this compound selectively and irreversibly depolymerizes DNA in bacterial cells (Reich, Shatkin & Tatum, 1961). It is therefore of significance that colourless mutants were 2·7–3·5 times, and mutants with orange colonies 33–45 times more vulnerable to the action

of this compound than the parent culture. It may be suggested that some alteration in the DNA of mutants with impaired respiration made the cells more vulnerable to the action of mitomycin C, and that these alterations are more fundamental in mutants with orange colonies as compared to colourless mutants.

Table 2. *Effect of Mitomycin C and Actinomycin C on the growth and nucleic acid synthesis of* Staphylococcus afermentans*

Compound, μg./ml.	Incubation time, min.	Turbidity, E_{660}	RNA (orcinol reaction) E_{660}	DNA (diphenylamine reaction) E_{540}
Control	0	0·190	0·411	0·091
	75	0·265	0·481	0·113
	150	0·337	0·578	0·132
Mitomycin C, 0·1	0	0·190	0·411	*0·091*
	75	0·270	0·487	*0·086*
	150	0·342	0·577	*0·088*
Control	0	0·171	0·399	0·085
	90	0·261	0·477	0·109
	180	0·369	0·581	0·136
Actinomycin C, 0·1	0	0·171	*0·399*	0·085
	90	0·209	*0·359*	0·092
	180	0·259	*0·372*	0·109

* The content of RNA and DNA in the microbial cells are given per 100 ml. of the culture. Turbidity E_{660} is proportional to the dry weight of bacteria. The figures give the average data of 3 series of experiments. Growth at 37° in Erlenmeyer's flasks. To 180 ml. of nutrient broth were added 20 ml. of 18 hr. culture of bacteria, and kept at 37° for 90 min. At this time compounds were added and the first sample taken, marked as '0 time'. For each analysis two samples of 50 ml. each were taken from two duplicate flasks, and mixed to make 100 ml.

Table 3. *Minimal growth inhibitory concentrations (μg./ml.) of various compounds in nutrient broth for* Staphylococcus afermentans *and its mutants**

Compound	Parent	Mutant 42 (orange)	Mutant 22 (orange)	Mutant 19 (colourless)	Mutant 44 (colourless)
Mitomycin C	0·0500	0·0015	0·0011	0·0140	0·0180
Degranol	210	22	28	25	29
Myleran-mannitol	4000	620	520	620	410

* The conditions of assays were described by Gause & Kochetkova (1962).

Differences in the DNA-protein relationship of normal and mutant organisms. The extraction of DNA from bacterial cells by Schmidt and Thannhauser procedure (in the modification described by Spirin *et al.* 1957; see Methods) showed that the average activity of extracts (D_{595}–D_{650}) in the parent culture attained 0·262, in mutant 19 (colourless) 0·094, in mutant 44 (colourless) 0·090, in mutant 42 (orange) 0·036, and in mutant 22 (orange) 0·052. In other words the capacity of DNA for extraction from a complex with proteins was decreased in colourless mutants by 2·8–2·9 times, and in mutants with orange colonies by 5·0–7·3 times.

Our observations refer to mild separation of DNA from proteins by extraction at pH 8 by 10% NaCl, as prescribed by Schmidt & Thannhauser (1945). In distinction from this, an acid hydrolysis by 0·5 N-HClO at 70° for 20 min. (Ogur & Rosen, 1950)

completely separates DNA from proteins in normal and mutant cells of *Staphylococcus afermentans*, and differences between these cultures are no longer observed. It is difficult at present to interpret these observations, but it is interesting that Kirby (1961) found recently some differences in the DNA-protein complexes of normal and cancer cells.

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Transamination Reactions in Uredospores of *Puccinia helianthi*

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SUMMARY

Various transamination reactions were observed in germinating uredospores of *Puccinia helianthi* Schw. The localization and kinetic properties of glutamate–aspartate transaminase were examined. The presence of these transaminase enzymes in the uredospores suggests that the uredospore could synthesize a large number of amino acids.

INTRODUCTION

Since the discovery of enzymic transamination in pigeon breast muscle by Braunstein & Kritzmann (1937) many transamination reactions have been studied in various organisms including fungi. In *Neurospora crassa* there are at least four transaminases which show specificity for α-ketoglutarate (Fincham & Boulter, 1956) and α-keto-β-methylvalerate and α-keto-isovalerate have been shown to transaminate readily with leucine and phenylalanine (Wagner & Ifland, 1956; Wagner, Berquist & Karp, 1958). Sanwal (1958) showed that in cell-free extracts of *Fusarium lycopersici* some 10 or 11 amino acids underwent transamination with α-ketoglutarate. In *Blastocladiella emersonii* an alanine glyoxylate transaminase has been demonstrated and purified (McCurdy & Cantino, 1960). *Saccharomyces cerevisiae* can transaminate several amino acids (Bigger-Gehring, 1955), and it seems likely that there is more than one transaminase responsible for these reactions (Sentheshanmuganathan, 1960). The present paper gives the results of an examination of the scope of transamination reactions in germinating uredospores of the obligate rust parasite *Puccinia helianthi* Schw.

METHODS

Organism and enzyme preparations

Uredospores were collected from plants of *Helianthus annuus* var. Foundation (seeds of which were kindly given by Co-operative Vegetable Oils Ltd., Manitoba, Canada) inoculated with *Puccinia helianthi*. The spores were placed in small dry vials (about 300 mg./5 ml. vial) which were tightly corked and stored at −10°. Under these conditions uredospores can be stored for several months with little loss in viability (Sackston, 1960).

Uredospores were brushed evenly over the surface of water-filled Visking tubes, which had previously been soaked in warm water for 6 hr., and the spores allowed to germinate in a covered glass tank for 20 hr. To aid the germination a phenol

solution (5×10^{-4} M) was introduced into the moisture-saturated atmosphere on strips of filter paper. Phenol stimulates germination and in common with coumarin (Farkas & Ledingham, 1959) is sufficiently volatile to be active across an air gap. The resulting mat of mycelium was carefully removed, rinsed in tapwater, and blotted dry.

The particulate fraction was isolated by grinding the germinated spores and an equal weight of acid-washed powdered glass for 1 min. in a chilled mortar with a solution containing 0·6M-sucrose + 0·05M-potassium phosphate + 0·005 M-ethylenediaminetetra-acetic acid (EDTA), previously adjusted to pH 7·8 with N-NaOH. The homogenate was passed through several layers of moist butter muslin and clarified by centrifugation at 1000 *g* for 10 min. in an M.S.E. 'High-Speed 17' refrigerated centrifuge to remove coarse cell debris and nuclei. The particulate fraction was then sedimented from the supernatant fluid by centrifugation at 20,000 *g* for 30 min. The deposit was suspended in the extraction medium, resedimented and then suspended in a small volume of 0·6 M-sucrose in 0·05 M-phosphate buffer (pH 7·9).

Assay of enzyme activities

Transaminase activity was assayed by measuring quantitatively the appearance of the new amino acid on paper chromatograms. The reaction system contained 0·4 ml. enzyme preparation, 0·1 ml. (30 μmole) α-keto acid, 0·05 ml. (10 μmole) amino acid, 0·05 ml. (1 μmole) pyridoxal phosphate and potassium phosphate buffer (pH 8·0) to a final volume of 1·0 ml. Where DL-isomers were used the substrate concentration was doubled. A reference control was run with the same mixture but lacking the amino acid donor. Incubations were carried out at 34° and the reaction stopped by immersing the tubes in boiling water for 3 min. Denatured proteins were removed by centrifugation at 3000 *g* for 10 min. The amino acid components were separated by one-dimensional ascending paper chromatography on Whatman No. 1 filter paper by using either 80 % phenol or *n*-butanol + pyridine + water (3 + 2 + 1·5 by vol.) as solvents. The amino acids were located by spraying with 0·25 % ninhydrin in 95 % ethanol in water. The individual coloured spots were identified by their known R_f values carefully cut out and eluted overnight at 2° in 50 % ethanol in 0·025 M-phosphate buffer (pH 6·5). The absorption of the eluted colours at 570 mμ was determined. Background corrections were made by cutting a comparable section of paper from the reference control, eluting it, measuring the absorption of the solution at 570 mμ and subtracting this value from the absorption of the amino acid complex. Amino acid solutions of known concentration were subjected to this procedure and standard curves obtained for each amino acid.

A unit of enzyme activity is defined here as the amount of enzyme required to produce by transamination 0·1 μmole acid/hr./mg. protein.

The amount of protein in solution was calculated from the optical density of the enzyme preparations at 260 mμ and 280 mμ (Adams).

RESULTS

Range of transamination

Twenty-two amino acids were tested qualitatively and quantitatively for ability to transfer their amino group to α-ketoglutarate in the presence of a washed particulate fraction from germinated uredospores. It was not found necessary to dialyse

the extracts since preliminary experiments showed that there were no free amino acids present which would interfere with the chromatographic analysis. Positive transamination occurred with phenylalanine, γ-aminobutyric acid, arginine, asparagine, valine, tyrosine, aspartic acid, lysine, alanine, isoleucine, leucine, tryptophan and methionine (Table 1). With the exception of aspartic acid the transamination activities were low. In the case of methionine and tryptophan where extremely low transamination rates were obtained the results were always consistently higher (2–3 times) than the reference control. When the extract was dialysed for 18 hr. against de-ionized water at 1° the ability to transaminate with tryptophan and methionine was lost and with asparagine and leucine was decreased to trace values.

Table 1. *Transaminase activity of a particulate fraction from uredospores of* Puccinia helianthi

The reaction system contained: 10 μmole amino acid; 30 μmole potassium α-ketoglutarate; 1 μmole pyridoxal phosphate; 0·4 ml. enzyme suspension and 40 μmole potassium phosphate buffer containing 0·1 % (v/v) Nonidet P 40, pH 7·9. Total volume 1 ml., temp. 32°.

Amino group donors	Units of activity	Amino group donors	Units of activity
L-Lysine	2·50	L-Serine	—
L-Alanine	0·50	L-Histidine	—
L-Isoleucine	0·61	L-Aspartic acid	12·50
L-Leucine	0·57	L-Ornithine	—
Glycine	—	L-Valine	0·45
L-Citrulline	—	L-Methionine	0·20
L-Proline	—	L-Tyrosine	0·80
L-Phenylalanine	0·65	L-Cysteine	—
γ-Amino-butyric acid	0·70	L-Cystine	—
L-Arginine	0·46	L-Tryptophan	0·10
L-Asparagine	0·76	L-Threonine	—

Localization of transaminase activity

Since the particulate fractions used in these experiments were thoroughly washed in an isotonic medium to remove any cytoplasmic enzymes which may have adhered to the surface of particles during the extraction procedure, it is considered that the transaminase activity associated with the particles is most probably derived from particulate-bound enzymes. Furthermore, the glutamate–aspartate transaminase could be quantitatively released and solubilized from the particles by treatment with Nonidet P40 (Shell Chemical Co., Ltd., Glasgow) a non-ionic detergent. The ability of this detergent to free particulate-bound enzymes has previously been demonstrated for plant tissue (Smith, 1962). The soluble extracts were obtained by treating the particulate fraction with a 0·1% (v/v) solution of Nonidet in 0·6 M-sucrose + phosphate buffer and removing the particulate fragments by centrifugation for 30 min. at 20,000 *g*. A comparable fraction was similarly treated except the buffer did not contain Nonidet. The supernatant solutions were retained and the particulate deposits in each case taken up in a similar volume of buffer which contained 0·1 % Nonidet to ensure complete activation of the bound enzymes When the Nonidet was added prior to centrifugation the glutamate–aspartate transaminase was quantitatively released or solubilized from the mitochondria whereas in the control lacking Nonidet the enzyme remained firmly attached to

the mitochondria. Control experiments showed that the detergent did not inhibit or stimulate the enzyme.

Coenzyme requirements

The effect of pyridoxal phosphate (10 μg. free acid/ml. reaction mixture) on glutamate–aspartate transaminase was examined. The addition of coenzyme had little effect on the undialysed enzyme but it did increase activity by 20 % when the enzyme extract had been dialysed for several hours. Partial resolution of the transaminase into apoenzyme and coenzyme as a result of dialysis would account for this increase in activity.

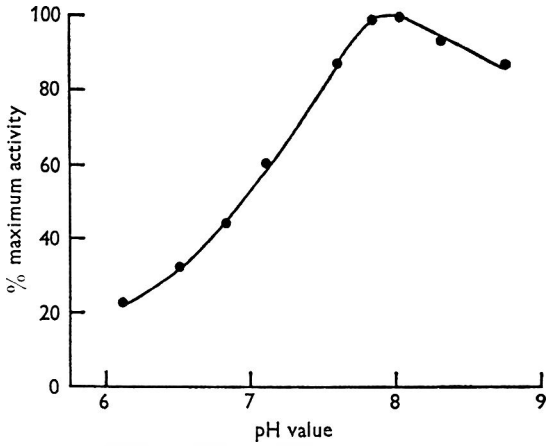

Fig. 1. The effect of pH on glutamate–aspartate transaminase. Reaction system as in Table 1. except for variation in pH value as indicated. Incubated at 32° for 30 min. Total volume 1 ml.

pH activity curve

The activity of glutamate-aspartate transaminase was examined as a function of pH value. Activities were estimated from the rate of glutamic acid formation under the conditions described for the enzyme assay. In these determinations the final buffer concentration was 0·05 M and the pH value of the reaction mixture was measured immediately after the assay. Phosphate buffer was used for pH 6·0 to 8·0 and borax + boric acid for pH 8·0 to 9·0. Initial reaction velocities are expressed as percentages of maximum activity. The activity curve is illustrated in Fig. 1 and shows that the enzyme has an optimum at pH 7·8–8·0.

Reversibility

The reversibility of the glutamate-aspartate transaminase reaction was demonstrated by the formation of aspartate from glutamate and oxaloacetic acid (forward reaction) and by the formation of glutamate from aspartate and α-ketoglutarate (reverse reaction). Progress curves of the forward and reverse reactions are shown in Fig. 2. The points on the curve were obtained by estimating glutamic acid and aspartic acid at each specified time. Equilibrium was reached in 3 hr. and this balanced system remained unchanged for a further 1 hr. The equilibrium was 60 % in favour of the formation of glutamic acid (Aspartate + α-ketoglutarate reaction).

Calculation of the equilibrium constant for the reactions gives a value of

$$K = \frac{(\text{aspartate})\,(\alpha\text{-ketoglutarate})}{(\text{glutamate})\,(\text{oxaloacetate})} = 2{\cdot}3 \text{ at pH } 7{\cdot}9 \text{ and } 32°.$$

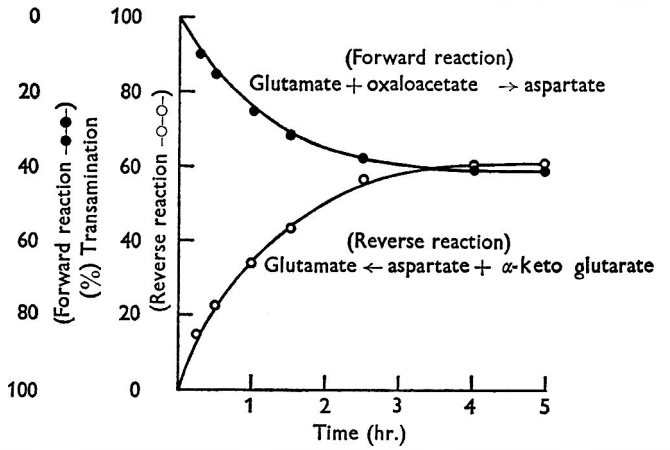

Fig. 2. Reversibility of glutamate–aspartate transaminase. Ordinate numbers on the right show percentage transamination measured by glutamic acid formation (reverse reaction (○)). Ordinate numbers on the left show percentage transamination measured by aspartic acid formation (forward reaction (●)). Reaction system contained: 10 μmole L-amino acid; 30 μmole keto acid; 1 μmole pyridoxal phosphate; 0·4 ml. enzyme extract and potassium phosphate buffer containing 0·1 % (v/v) Nonidet P40, pH 7·9. Total volume 1 ml.; temp. 32°.

DISCUSSION

The results of these experiments demonstrate unequivocally that a wide variety of transaminase reactions occur in germinating uredospores of *Puccinia helianthi* and that in common with plant and animal tissue (Smith, 1962; Hird & Rowsell, 1950) enzyme activity can be localized on a particulate fraction.

The reversibility of the glutamate-aspartate enzyme was demonstrated and the equilibrium constant at pH 7·9 and 32° was 2·3. This is in close agreement with the 2·2 obtained by Bigger-Gehring (1955) for the same enzyme from *Saccharomyces cerevisiae*. The uredospore enzyme showed optimum activity between pH 7·8 and 8·0. The effect of pyridoxal phosphate on the enzyme was small, though it seems that this compound is the coenzyme for all transaminase enzymes (Meister, 1957). The effect of pyridoxal phosphate on the dialysed enzyme suggests that some resolution into apoenzyme and coenzyme had occurred during the period of dialysis.

The presence of these transaminase enzymes in the uredospores would suggest that the uredospore could synthesize a wide variety of amino acids provided the corresponding keto acids and glutamic acid were available.

Several rust fungi can synthesize amino acids from extraneous carbohydrates (Kasting, McGinnis & Broadfoot, 1959; Staples, Burchfield & Baker, 1961; Reisener, McConnell & Ledingham, 1961). Reisener *et al.* (1961) germinated uredospores of *Puccinia graminis tritici* on a solution containing valerate-1-^{14}C and demonstrated the incorporation of the labelled carbon into several amino acids, in particular glutamic acid, γ-aminobutyric acid, glutamine and alanine. The high activity of

the C_1 in glutamate suggests that the valerate had undergone extensive β oxidation to acetyl CoA which was subsequently metabolized via the tricarboxylic acid (TCA) cycle. Reisener *et al.* (1961) have suggested that the γ-aminobutyric acid may result from the enzymic decarboxylation of glutamic acid. However, this may not be so, since there is apparently no glutamic decarboxylase present in *P. helianthi* (Smith, 1959) and furthermore, there is a decrease in glutamic decarboxylase activity in several different rust-infected host-parasite complexes, including susceptible wheat infected with *P. graminis tritici* (Smith, 1959).

In a comparative study of the metabolic capabilities of several obligately parasitic and saprophytic fungi (Staples *et al.* 1961), there is strong indication that the assimilate, in this case acetate-2-^{14}C, is also metabolized by way of the TCA cycle since the first free and protein amino acids to be labelled are all closely associated with the cycle. Although labelled leucine and methionine, which are not directly associated with the TCA cycle, did eventually accumulate in the free amino acid pool of the obligate parasite *Uromyces phaseoli*, they were never incorporated into protein. Conversely, short-term incubation with L-leucine-U-^{14}C and L-methionine-^{35}S did lead to the rapid incorporation of the labelled substance into the protein fraction. Further studies showed that *U. phaseoli* suffered a rapid loss in the mechanism for protein synthesis after wetting and this coupled with slow synthesis of leucine and methionine would account for the failure to detect labelling in the leucine and methionine in the protein fraction. Thus it would seem that the uredospores do have the mechanism for the synthesis of proteins but are restricted by their slowness or inability to synthesize the precursors of proteins.

The rapid synthesis of glutamic acid shown by uredospores of several rust fungi (Staples *et al.* 1961; Reisener *et al.* 1961) may be due to the activity of glutamic dehydrogenase since germinating uredospores of *Puccina helianthi* (Smith, 1959) contain a highly active glutamic dehydrogenase which catalyses the formation of glutamate from α-ketoglutarate and ammonia. This enzyme plays a very significant part in metabolism as it serves not only as a port of entry for ammonia but also as a link between carbohydrate and nitrogen metabolism. Further metabolism of the glutamic acid by transamination could involve extensive interconversion of the amino group giving rise to a wide variety of amino acids. The limiting factor in this hypothesis would undoubtedly be the availability of keto acids, in particular the keto acids remote from the TCA cycle.

The failure to detect labelled carbon in certain amino acids and slow uptake of labelled carbon in others (Staples *et al.* 1961; Reisener *et al.* 1961) does not necessarily imply that these amino acids were not formed. On the assumption that glutamic acid is synthesized by reductive amination of α-ketoglutaric acid and subsequently involved in a variety of transamination reactions, then the labelled carbon would not appear immediately in the new amino acid but in α-ketoglutaric acid. Hence a labelled amino acid would be formed only when the labelled carbon had been previously incorporated into the corresponding keto acid.

Since uredospores can incorporate labelled carbon into 10 or 12 amino acids from extraneous carbohydrate after prolonged incubation (Kasting *et al.* 1959; Staples *et al.* 1961; Reisener *et al.* 1961) and assuming that transamination plays a major role in their synthesis, it seems safe to deduce that the uredospores are able to synthesize the corresponding keto acids. The apparent inability to synthesize

certain amino acids and the relative slow synthesis of others may indicate that the parasite is dependent on the host for the supply of these amino acids or conversely on a supply of the corresponding keto acids. It has already been suggested that races of rust may differ in their specific amino acid requirements, in particular those amino acids which occur in very low concentrations (Samborski, Forsyth & Person, 1958; Samborski & Forsyth, 1960).

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Pulmonary Infection of Adult White Mice with the TE 55 Strain of Trachoma Virus

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SUMMARY

The effects of intranasal inoculation of mice more than 6 weeks old with the TE 55 strain of trachoma virus were studied. The histology and course of the pulmonary lesions produced are described. Infected mice showed a poor response of complement-fixing antibodies after infection. Precipitating antigens were detected in infected mouse lungs.

INTRODUCTION

Thygeson & Nataf (1958) stated, 'It is now generally agreed that only primates are susceptible to experimental infection with trachoma virus'. Since then Giroud, Renoux & Nataf (1958) have reported pulmonary hepatization in mice after intranasal inoculation of material from trachomatous conjunctivae; Hurst & Reeve (1960) reported passage of the TE 55 strain in mouse brain; and Bernkopf (1959) reported the susceptibility of young mice to infection after intranasal inoculation with the TE 55 and Dari strains of trachoma virus. Bell, Snyder & Murray (1959) showed a rapid toxic effect of certain strains after intravenous injection into adult mice, but this phenomenon did not depend upon the growth of virus. These workers were chiefly interested in the mouse either as a means of growing the virus or as an experimental system for neutralization tests. For the second purpose a lethal effect of intranasal inoculation was required. Bernkopf (1959) found that 9-day-old mice were highly susceptible, three-week old mice much less susceptible, and six-week old mice insusceptible to intranasal inoculation with the TE 55 strain. Insusceptibility in this sense means that the mice did not die.

The experiments reported here were made to examine the effects of intranasal inoculation of older, and therefore 'insusceptible' mice with the TE 55 strain of trachoma virus. It was considered that these mice would either be totally insusceptible, in which case the system could be studied as an example of host resistance developing with age, or partly susceptible, in which case lesions would develop which would not lead to death, and would presumably resolve. Preliminary observations showed that older mice were, in fact, partly susceptible, and that they would provide a system in which the pathogenesis and immunology of trachomatous infection could be studied in a non-primate host.

METHODS

Virus. The TE 55 strain of trachoma virus (T'ang *et al.* 1957), propagated in chick embryo yolk sac, was provided by Dr L. H. Collier (The Lister Institute, London). At the beginning of these experiments the virus was in its 37th passage,

at the end in its 48th. The methods of propagation and storage were described previously (Watkins, 1961). Infected yolk sac homogenates were inoculated on to blood agar and into thioglycollate medium (Brewer, 1940) to examine for bacterial contamination.

Estimation of infectivity. The infectivity of virus suspensions is expressed as the Mean Survival Time (MST), in days, of four to six chick embryos after injection of 0·2 ml. of suspension into each yolk sac at 7 or 8 days of incubation. The MST is approximately inversely proportional to the infectivity of the suspension inoculated (Watkins, 1961). The most infective suspensions gave an MST of 4–5 days, the least an MST of 13–14 days, by this technique.

Mice. An inbred strain of Swiss albino mice was used. All mice weighed 20 to 30 g., and were over 6 weeks old. Two or three drops of infected yolk sac homogenate (0·05–0·075 ml.) were inoculated intranasally under ether anaesthesia. Mice were killed by dislocation of the cervical vertebrae.

Mouse lung homogenates were prepared by grinding the lungs in 2 ml. of sucrose + potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950) containing 50,000 μg. streptomycin/ml. in a Griffiths tube. The homogenate was centrifuged at 400 ***g*** for 10 min. and the supernatant fluid used in experiments. This supernatant fluid will be referred to as MLH (mouse lung homogenate). 0·2 ml. of MLH was injected into four to six 7 day yolk sacs for infectivity studies. Eggs were candled daily for 14 days.

Histology. Tissues for histological examination were fixed in Zenker-formol solution or 5 % (v/v) formol-saline and stained with Giemsa's stain or haematoxylin and eosin. Homogenates examined for elementary bodies were stained with Giemsa stain (G. T. Gurr, London).

Antisera. The antiserum designated R1/3 was prepared by five weekly intramuscular injections of 1 ml. of stock infected yolk sac homogenate (MST 4–5 days) into a rabbit. A booster dose was given 5 months later and blood was taken 1 week after the booster dose. The serum was stored at $-20°$.

Mouse sera were obtained by bleeding from the tail or the jugular vein under light ether anaesthesia. After separation the pooled sera were preserved by the addition of thiomersalate to 1/10,000 (w/v), or sodium azide to 0·1 % (w/v) or by freezing at $-20°$. All sera were inactivated for 30 min. at 56°.

Preparation of antigens. Antigens were prepared in one of two ways.

(1) Yolk sacs rich in elementary bodies were homogenized in 10 ml. phosphate-buffered saline (PBS, Dulbecco & Vogt, 1954) per yolk-sac. The homogenate was shaken with an equal volume of ether and the mixture placed at 4° overnight. The lower red layer was centrifuged at 400 ***g*** for 10 min., the deposit washed three times in 2 ml. of PBS and the washings added to the supernatant fluid, which was centrifuged at 10,000 ***g*** for 30 min. This deposit was resuspended to the original volume in PBS and centrifuged at 400 ***g*** for 10 min. and the supernatant from this at 10,000 ***g*** for 30 min. After a third cycle of centrifugation at 400 ***g*** for 10 min. and 10,000 ***g*** for 30 min. the final deposit was resuspended in CFT diluent (referred to below) at 3 ml./original yolk sac. Smears of this antigen preparation showed more than 100 elementary bodies/microscope field at a magnification of $\times 1700$. Electron microscopy of a suspension prepared in this way was kindly undertaken by Dr D. Kay (Sir William Dunn School of Pathology). A drop of virus suspension

was placed on a formvar coated grid, fixed in formaldehyde vapour, washed in water and examined after shadowing with palladium. Plate 1, fig. 1, shows intact virus particles.

(2) Some antigens were prepared by the method of Nigg, Hilleman & Bowser (1946) which involves phenolizing an infected yolk sac suspension with 0·5 % phenol for four weeks at 37°. In our experiments the antigen was shaken with phenol at 37° for 2 weeks.

Control antigens for each type of virus antigen were prepared from normal yolk sacs of the same age. It is known that sera from cases of lymphogranuloma venereum will fix complement with phenolized trachoma antigen (Woolridge, Jackson & Grayston, 1960). Two LGV sera (kindly provided by Professor C. F. Barwell, The London Hospital) gave titres of 1/256 and 1/512 with phenolized antigen in the CF test described below. They did not fix complement with controls made from normal yolk sacs.

Complement-fixation tests. These were performed in cups in Perspex trays (Salk pattern haemagglutination trays). The unit volume was 0·02 ml. delivered from a calibrated dropping pipette. The minimal haemolytic dose (MHD) of complement was determined for 100 % haemolysis in the presence of antigen. Twofold serial dilutions of antiserum were mixed with undiluted antigen and 2 MHD of complement. Fixation took place at 4° for 18 hr. followed by 20 min. at room temperature. After addition of the haemolytic system (2·5 % (v/v) sheep red cells and 3–4 MHD of haemolytic serum) incubation was continued at 37° until haemolysis was complete which took 1½-2 hr. Controls of ½ MHD, 1 MHD and 2 MHD of complement and serum and antigen controls were included in each batch of tests. The guinea-pig complement had been preserved by Richardson's method (1941) (Burroughs Wellcome & Co., London). Horse anti-sheep cell serum was preserved with glycerol (Burroughs Wellcome & Co., London). All dilutions were made in barbitone CFT diluent made up from commercially obtained tablets (Oxoid, England).

Agar gel precipitation (Ouchterlony, 1948). Perspex moulds were made consisting of six pegs arranged around a central peg. Holes were bored through the centres of the pegs. Each peg was 1·0 cm. in diameter, and the distance between any two adjacent pegs was 0·5 cm. The mould was placed in a flat-bottomed Petri dish 10 cm. in diameter and 10 ml. of 2 % (w/v) agar in 0·9 % (w/v) NaCl with 1/10,000 thiomersalate were poured in. The mould was removed when the agar had set, 0·1 ml. of antiserum or antigen solution was placed in the cups and the plate incubated at 37° in a humid atmosphere. Lines of precipitation developed in the agar within 2 days; further incubation produced no change in the number of lines.

RESULTS

Absence of infectivity and antigens in lungs of control mice

It is well known that latent infection with several viruses may occur in laboratory mice. To test for the presence of such viruses normal yolk sac homogenate was instilled intranasally into normal mice; the lung homogenate from these mice was then instilled into fresh mice and so on for five passages. The homogenates from control mice were examined for infectivity by inoculation into yolk sacs and for the presence of antigen precipitating with serum R1/3 in agar. Neither infectivity

nor precipitating antigen was detected in these homogenates after various intervals between intranasal inoculation and removal of the lungs (Table 1). Homogenates of normal mouse lungs, normal mouse lungs after intranasal inoculation of normal yolk sac homogenate, and normal mouse lung after intranasal inoculation of normal mouse lung homogenate were examined on several other occasions for yolk sac infectivity and the presence of precipitating antigen. The results were uniformly negative. It was concluded that any 'elementary body virus' and precipitating antigen recovered from lungs after intranasal inoculation of yolk sac homogenate containing the TE 55 strain of trachoma virus must have been derived from inoculated virus and not from an awakened latent infection.

Table 1. *Presence or absence of lines in agar produced by mouse lung homogenate and rabbit antiserum* R1/3 *after various inocula intranasally*

Inoculum	Time between inoculation and removal of lungs (days)	No. of mice	No. of lungs positive	No. of lungs negative
Normal yolk sac homogenate	7	6	0	6
	11	2	0	2
	14	6	0	6
Virus TE55 infected yolk sac homogenate (MST* between 4 and 5 days)	1	7	3	4
	2	1	1	0
	3	1	1	0
	4	1	1	0
	7	4	1	3
	11	2	1	1
	14	2	2	0
	28	1	1	0
Normal mouse lung homogenate (passage)	7	5	0	5
	14	6	0	6
	20	6	0	6
	52	4	0	4
Passage infected (virus TE55) mouse lung homogenate	4	3	1	2
	10	4	4	0
	14	4	4	0
	18	1	1	0
	22	1	1	0
No inoculum	0	10	0	10

* Mean survival time.

Table 2. *Relation between lethality of virus TE55 for chick embryos after yolk sac inoculation, and mice after intranasal inoculation*

MST of inoculum in yolk sac (days)	No. of mice inoculated	No. and percentage of mice dying of pneumonitis
4·4	27	14 (51·8 %)
4·6	140	3 (2·14 %)
4·7	60	0
> 5	> 400*	0

* At various times throughout these experiments.

Lethality of virus for mice after intranasal inoculation

Table 2 shows the relation between the infectivity of TE 55 virus for the yolk sac, expressed as the mean survival time (MST) of chick embryos, and the percentage of mice dying of pneumonitis after intranasal inoculation. It is apparent that deaths occurred only after the administration of inocula with high infectivity for chick embryos. All the fatally infected mice died between 2 and 4 days after infection. Post-mortem examination showed massive consolidation of both lungs, and virus was recovered from several lungs by inoculation into the yolk sac. The mice which survived inoculation showed for the most part no signs of illness; some of those which received higher doses had staring fur for a few days, and sometimes râles could be heard by holding the mouse close to the ear.

Histology of lesions

The massively consolidated lungs in fatally infected mice showed acute inflammation (Pl. 1, figs. 2 & 3). Typical inclusion bodies were seen occasionally. It was impossible to discern which cells contained these bodies, although in some cases they were situated in the alveolar wall (Pl. 2, fig. 4*a*, *b*). The inclusion shown was photographed at two levels to show the elementary bodies which it contained. At the periphery of this inclusion larger bodies can be seen, conforming to the description of trachomatous initial bodies first given by Lindner (1910). The inclusion bodies did not differ morphologically or in staining properties from trachoma inclusions in yolk sac entodermal cells and HeLa cells.

In mice which received a sublethal dose the size of the lesion varied with the infectivity of the inoculum. Inocula with a chick embryo MST of 5–6 days produced local consolidations sometimes a few millimetres in diameter, sometimes affecting a single lobe in its entirety. The numbers and sizes of the lesions were too variable to be used for virus titration in the way psittacosis virus has been titrated in mouse lung (Rudd & Burnet, 1941). After inocula having chick embryo MST values greater than 7 or 8 days, lesions visible to the naked eye were generally not seen; when they were seen they were small. The earliest histological lesions were seen 2 days after infection in an experiment with an inoculum whose MST value for chick embryos was 5 days; they consisted of areas about 70 μ in diameter of infiltration with polymorphonuclear leucocytes. There was also some generalized capillary dilation. By the 3rd day the areas had become confluent, and gave the low-power appearance shown in Pl. 2, fig. 5. This photograph shows peribronchial infiltration, and is taken from a set of serial sections which demonstrated that the bronchioles in the centre of the inflammatory area opened into normal alveoli. In this case, therefore, the picture is that of a bronchiolitis; in other mice the lesions affected only the alveoli. The difference is presumably due to variation in the distance to which the inoculum penetrated as a result of inspiration. On the 4th day the vessels in the areas of inflammation were surrounded by zones of mononuclear cells (Pl. 2, fig. 6). Resolution of the lesions was completed between the seventh and ninth days, but some perivascular cuffing with mononuclear cells persisted until at least the 28th day. After resolution of the acute lesions there was no evidence of tissue destruction or fibrosis or lymphocytic infiltration characteristic

of ophthalmic trachoma. Apart from enlargement of hilar lymph nodes, which persisted for 2–3 weeks after infection no other organs showed any abnormalities.

Recovery of virus from mouse lung after intranasal inoculation of infected yolk sac homogenate

Infective virus, indistinguishable morphologically from trachoma virus, was recovered from lungs by yolk sac inoculation several times at intervals from 1 hr. to 7 days after intranasal inoculation of yolk sac homogenates of infectivities ranging from MST 4·4 days to MST 8·0 days. The infectivities of MLH ranged from MST 7·5 days to MST 12·5 days in all these experiments. The infectivities of lungs from mice killed at intervals up to 7 days after receiving the same inoculum showed no significant increase, which suggested that virus was either remaining static in the lung, or was being removed at a rate which kept pace with its replication. No infectivity was detected 14 days or more after inoculation and recovery of virus was not uniformly successful in the period up to 7 days after inoculation.

Presence of viral antigen in lungs

Soluble antigens were detected in infected lungs by diffusion in an Ouchterlony plate against a rabbit antiserum (R1/3) prepared by repeated intramuscular injections of infected yolk sac homogenate. Homogenates prepared from normal mouse lungs after instillation of normal yolk sac or normal lung homogenates never gave precipitation lines with this or any other anti-trachoma virus serum. Lines were not seen after inoculation of virus suspensions whose MST was greater than 6 or 7 days, and even after the inoculation of suspensions of higher infectivity their appearance was not constant (as Table 1). They were most frequent when homogenates were made from lungs showing massive consolidation; lines were given by a homogenate prepared 14 days after infection in an experiment in which half the mice died of pneumonitis. The antigen persisted for several weeks; lines were produced with a homogenate prepared 4 weeks after inoculation of virus with an MST of 4 days. The pattern of lines was constantly a single line near the antigen cup with a less well-defined complex of one to three lines near the antiserum cup. The lines were also produced with a second rabbit antiserum prepared by injection of infected yolk sac homogenate.

Serial passage of virus in mouse lung

Two passage series were attempted. The first was started by intranasal inoculation of six mice with virus of MST 5 days. Passage of infected lung homogenate was undertaken at intervals of 7–14 days except for an interval of 22 days between the eighth and ninth passages. Six mice were inoculated at each passage, and homogenates for further passage were made from two to six mice in each passage. The mice of all passages remained clinically well, except for one mouse left for observation out of the fifth passage, which died 18 days after inoculation with patchy pneumonitis of both lungs. Apart from the homogenates of the first two passages virus was not recovered from the lungs by a single passage of the homogenate in yolk sacs, which meant that there was no direct evidence that virus was being passed. There was, however, some indirect evidence. First, antigens were detected

in homogenates of all passages after the first four (which were not examined), by precipitation in Ouchterlony plates. The precipitation lines were identical with those described in the preceding section. Secondly, patches of pneumonitis were observed in mice of the second, fifth and ninth passages, and one mouse of the sixth passage showed moderate perivascular and peribronchial accumulations of cells of the kind described in the section on histology.

The second series was carried on for five passages. The first passage, of virus suspension with an MST of 4·4 days, killed half the mice in 3 days, and the second passage was made with homogenate of consolidated lungs. Precipitation lines identical with those of the first series were given by homogenates of lungs of all the passages. Virus was recovered from the first passage homogenate, by single passage yolk sac inoculation, but not from the subsequent passages. A control passage series started with homogenate of normal mouse lung, and continued for five passages, was negative in each passage for yolk sac infectivity (determined by a single passage) and the presence of precipitating antigens.

Table 3. *Complement-fixation titres of mouse sera after intranasal inoculation of trachoma virus TE55*

MST of inoculum (days)	No. of mice	No. dead with pneumonitis	Pulmonary consolidation in survivors	No. of mice in pool	Titre of serum	Time after inoculation (days)	Antigen*
4·6	140	3	+	7	< 1/8	3, 6, 9, 21	P
					1/8	12, 15, 18, 28, 30	
				3*	< 1/8	6, 9	P
					1/8	3, 12, 15	
Not determined	24	3	+	5	< 1/8	7, 14, 28	E
					1/64	21	
5·2	54	0	±	3	< 1/8	2, 4, 6, 8, 10, 12, 14	P
4·7	60	0	+	4	< 1/8	14	E

* Sera taken from mice which were clinically ill 3 days after inoculation.
P, phenolized antigen; E, etherized antigen.

Complement-fixation tests on sera from infected mice

Table 3 shows that pooled sera gave low titres of complement-fixing antibody against the two antigens used. Higher titres were obtained from mice surviving an inoculum which killed some of their fellows, but only in one pool (taken 21 days after infection) was the titre greater than 1 in 8. Pooled sera with titres of 1 in 8 occurred 12 days or more after infection, with the exception of one pool which gave a titre of 1 in 8, 3 days after infection. The significance of this is uncertain, since pools made 6 and 9 days after infection in the same experiment were negative.

DISCUSSION

It is clear that the TE 55 strain of trachoma virus will grow in the lungs of mice older than 6 weeks, although the type of cell in which growth occurs is at present unknown. After intranasal inoculation of Indian ink all the ink is taken up by

macrophages within 24 hr. (Watkins, unpublished). If trachoma virus is taken up in the same way it is possible that growth occurs within these macrophages. The inclusion bodies seen in mouse lung resembled trachoma inclusion bodies in conjunctival epithelium, so that the method of virus replication in a host far removed taxonomically from the natural host is, morphologically at least, of the same kind. It was surprising that inocula containing about 10^9 elementary bodies produced so few inclusion bodies, even in massively consolidated lungs. This may have been due to absence of infectivity of most of the particles, since the LD50 of this strain for chick embryos by the yolk sac route was of the order of 10^3 to 10^4 elementary bodies. An alternative explanation, that the particles successful in producing inclusion bodies were genetically different from the unsuccessful ones, is made unlikely by the failure of repeated passage to produce an increase in virulence. Material which killed chick embryos in more than 6 days rarely produced naked-eye or histological lesions. This suggests that the production of lesions depends on a balance between destruction of virus and its successful establishment within a cell.

The pathogenesis of the acute inflammation, which resembles that occurring in the early stages of human conjunctival infection, is still obscure. The extent of the reaction seemed out of proportion to the small numbers of inclusion bodies present. A simple reaction to inert foreign material can be excluded, since nothing similar is seen after heavy inocula of Indian ink. Three explanations are possible. The reaction may be provoked by the release of toxic elementary bodies from cells in which the virus is growing, Growth of virus may be accompanied by the production of a soluble toxin, as suggested by Mitsui (1954) and others. The detection of diffusible antigens by gel precipitation gives some support to this idea, since it shows that some kind of soluble material is associated with the growth of virus. The third explanation is based on the finding by Bell *et al.* (1959) that some strains of trachoma virus produce a rapid toxic death in mice after intravenous injection of washed elementary bodies. It is possible that intranasal inoculation of a large number of elementary bodies may itself produce an inflammatory reaction. On this view the growth of virus alone would be responsible at the most for only a small proportion of the reaction. At present it is impossible to say which of these three explanations is correct, or whether more than one mechanism may be involved.

The low titres of complement-fixing antibody against particulate (etherized) and phenolized antigen, together with the late appearance of detectable antibody, suggest that this type of antibody may have little to do with the process of resolution of the inflammation. Mice are capable of producing high titres of complement-fixing antibody (up to 1/5120) after intranasal infection with organisms of the pleuropneumonia group (Lemcke, 1961), so the low titres obtained after trachomatous infection may mean that trachoma virus is a relatively poor antigen for mice. Low titres of complement-fixing antibody are usual after infection of human beings with members of the psittacosis–lymphogranuloma group of viruses; it is difficult to ascertain whether this is due to a poor host response, or to deficiencies in the antigens used in complement-fixation tests.

The failure to recover infective virus from the lungs of passage mice when there was circumstantial evidence that their lungs were infected may perhaps be explained by the fact that two completely different criteria of infectivity were being compared. On the one hand, in trying to detect virus by yolk sac inoculation in these experi-

ments enough virus had to be present to kill the embryo within 14 days; on the other hand, to infect mouse lungs with mouse-lung passaged material enough virus had to be present to give rise to detectable diffusible antigen and to occasional small patches of pneumonitis. If the second method was more sensitive than the first the failure to recover virus from passage lungs can be explained. However, the possibility cannot be excluded that continued passage in mouse lung produced a strain of virus with altered properties of growth in yolk sac entoderm.

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EXPLANATION OF PLATES 1 & 2

Fig. 1. Electron micrograph of etherized suspension of trachoma virus TE55. Palladium-shadowed (× 50,000).

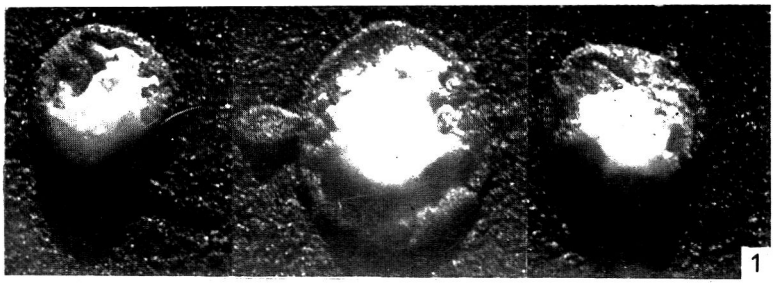
Fig. 2. Area of inflammation in mouse lung 3 days after intranasal inoculation with trachoma virus TE55 (× 450).

Fig. 3. Area of inflammation in mouse of fifth passage series (× 450).

Fig. 4. Trachoma inclusion body in same lung as that shown in Fig. 2, photographed at two different levels (× 1500).

Fig. 5. Mouse lung showing foci of inflammation 3 days after intranasal inoculation of trachoma virus TE55 (× 40).

Fig. 6. Perivascular infiltration of mononuclear cells into pneumonitic area in mouse lung 4 days after intranasal inoculation of trachoma virus TE55 (× 200).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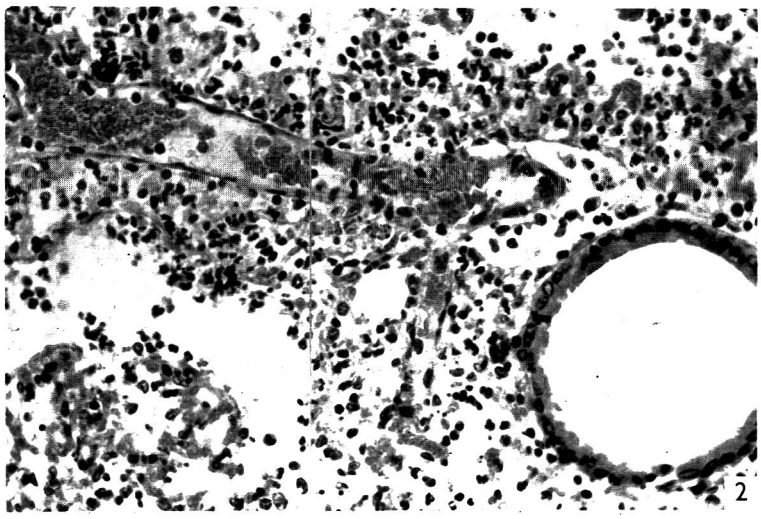
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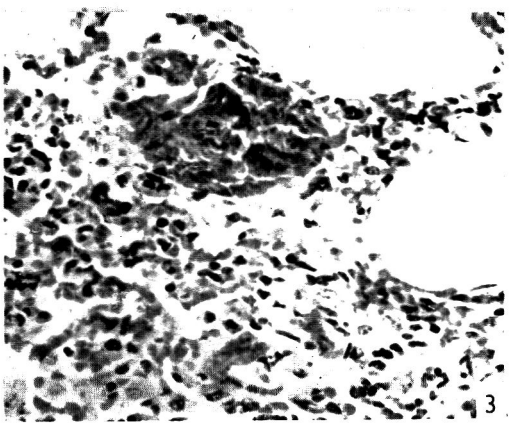
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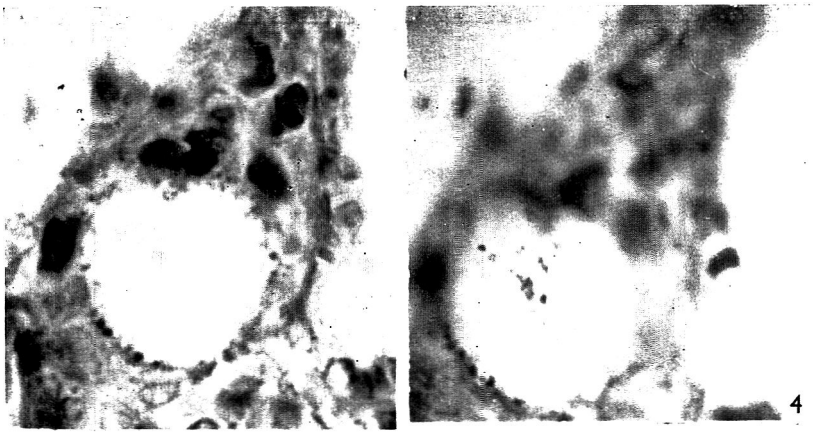
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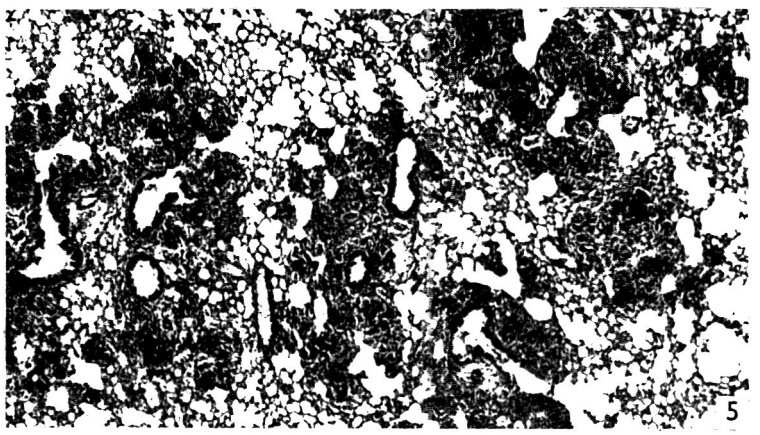
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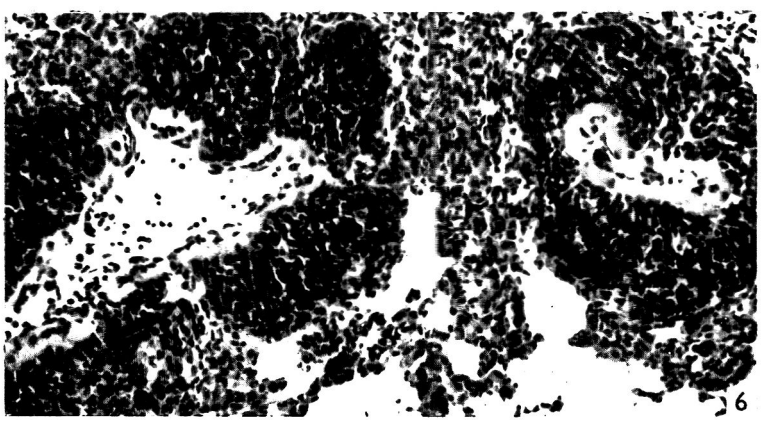
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J. gen. Microbiol. (1963), **30**, 53–58
With 1 *plate*
Printed in Great Britain

Factors which Affect the Size of the Organisms and the Optical Density of Suspensions of *Pseudomonas aeruginosa* and *Escherichia coli*

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(*Received* 24 *April* 1962)

SUMMARY

When water-washed organisms of a strain of *Pseudomonas aeruginosa* were exposed to solutions of NaCl or sucrose, they shrank in size and became more dense. The optical density of suspensions increased rapidly. Pre-treatment of the organisms with various cations, surface-active agents and heat and cold, affected the reaction, and the organisms then behaved differently toward NaCl and sucrose. Some but not all of the effects observed with *P. aeruginosa* were also observed with a strain of *Escherichia coli*.

INTRODUCTION

Mager, Kuczinski, Schatzberg & Avi-Dor (1956) observed that the turbidity of water suspensions of many Gram-negative bacteria increased when the osmotic pressure was increased by the addition of either electrolytes or non-electrolytes. The magnitude of the effect varied with the age, the degree of viability and the pH. Gram-positive bacteria did not show the turbidity change. Avi-Dor, Kuczinski, Schatzberg & Mager (1956) studied the kinetics of the turbidity changes in suspensions of *Pasteurella tularensis*. The initial increase was rapid, caused by the loss of water from the organisms, which decreased in size. This was followed by a decrease in turbidity and the rate of this decrease was affected by compounds which altered the metabolism of the organisms. In the present work, turbidity changes were studied in suspensions of a strain of *Pseudomonas aeruginosa*, an organism very sensitive to osmotic effects, and in a strain of *Escherichia coli*.

METHODS

A strain of *Pseudomonas aeruginosa* which has been maintained in this laboratory for 15 years, was grown at 34° for 24 hr. in Difco nutrient broth. The organisms were centrifuged down and washed twice with distilled water. In this process they lost potassium, an average of 5·7 μmole/40 ml. of wash water, but were subsequently able to take up potassium when incubated with potassium salts. The determinations of potassium were made with a flame photometer. One ml. of final suspension in water was placed in each of a series of test tubes. Various compounds were added dissolved in 0·1 or 0·2 ml. and allowed to react with the organisms for 15 min. at 23°. Water was then added to a final volume of 10 ml. and the initial optical density (O.D.) read at 490 mμ in a photocolorimeter. Solid NaCl, sucrose and other substances

were then added so that their final concentrations were 0·1–0·2 M and the O.D. read as soon as solution was complete, usually within 40 sec. During this time the maximal increase in O.D. had already occurred, and in fact the increase could be detected by the naked eye within a few seconds after addition was made. The O.D. was then measured at intervals. In other experiments various compounds were added after the O.D. had been increased by NaCl, sucrose, etc., and the rate of change as compared with the control was determined. The values are expressed as follows: a standard curve was obtained with different volumes of a suspension of untreated organisms. When the initial O.D. of an experimental series gave a reading corresponding to 1·0 ml. organisms in the standard curve it was given the value of 1·0, when the O.D. corresponded to 2·0 ml. organisms in the standard curve it was given the value of 2·0.

Avi-Dor *et al.* (1956) showed that the increase in optical density is the result of a decrease in organism size caused by water loss. Electron-microscope pictures taken of control organisms and others which had been exposed to 0·15 M-NaCl confirmed the decrease in size and showed an increase in density consistent with water loss (Pl. 1).

The effect of pre-incubation of Pseudomonas aeruginosa *with potassium salts on the change in O.D. caused by the addition of 0·1 M-NaCl or 0·2 M-sucrose*

Table 1 shows the effects of allowing the organisms to react with 0·05 M-KCl for 15 min. before dilution and addition of either NaCl or sucrose. Two effects can be seen. (1) The initial O.D., i.e., before addition of salt or sucrose, was decreased by

Table 1. *The effect of pre-incubation of* Pseudomonas aeruginosa *suspensions with 0·05 M-KCl on the increase in optical density (O.D.) values produced by 0·1 M-NaCl or 0·2 M-sucrose*

Pre-treatment	Agent	O.D. values		Agent	O.D. values	
		Initial	Zero time		Initial	Zero time
None	NaCl	0·99	1·72	Sucrose	0·99	1·75
KCl	NaCl	0·95	1·32	Sucrose	0·95	1·34

pre-incubation with KCl. This decrease was small but consistent and indicates an increase in size of organism as the result of the uptake of K^+ accompanied by water. (2) The increase in O.D. following the addition of salt or sucrose (zero time O.D.) was diminished by the pre-incubation with KCl. Apparently an increase in intracellular potassium partially protected the organisms against the subsequent osmotic effect. That the pre-incubation was necessary is shown by the fact that KCl added to the organisms immediately before dilution and addition of sucrose had little effect on the zero time O.D.

Since potassium was taken up by the organisms when they were suspended in water it was of interest to determine whether it would be taken up by organisms suspended in sucrose. Sucrose was added to the diluted suspension of organisms, the O.D. read, 0·2 ml. of 0·5 M-KCl added, and the O.D. read again after 2 min. There was a marked decrease in the O.D. reading, indicating an increase in organism size. Direct measurement of potassium uptake was not possible because of the sucrose, but the only reasonable mechanism to explain the effect is that K^+ ions

accompanied by water entered the cell. Table 2 compares the effect of potassium with other monovalent cations which also increased the organism size, though at a slower rate.

The entrance of cations into the organisms is probably the result of an active process. Table 3 shows the evidence for this in the case of potassium. Potassium phosphate solution (pH 7·4), was pre-incubated with the organisms for 15 min. in the presence and absence of 40 μg. KCN; the suspensions were then diluted and NaCl added. Cyanide prevented the effect of the potassium phosphate, presumably by inhibiting some metabolic activity necessary for the transport.

Table 2. *The change in optical density* (O.D.) *values 2 min. after the addition of 0·2 ml. of 0·5* M*-solutions of various monovalent cations to* Pseudomonas aeruginosa *organisms suspended in 10 ml. of 0·2* M*-sucrose*

Salt added	Change in O.D. value
None	0
LiCl	−0·26
NaCl	−0·30
KCl	−0·34
RbCl	−0·20
CsCl	−0·20

Table 3. *The effect of pre-incubation of* Pseudomonas aeruginosa *suspension with 0·05* M*-potassium phosphate solution (pH 7·4) with and without* 40 μg. *KCN on the increase in optical density* (O.D.) *value produced by 0·1* M*-NaCl*

Pre-treatment	O.D. values		
	Initial	Zero time	60 min.
None	1·00	1·70	1·49
K phosphate	0·81	1·09	0·86
KCN	0·87	1·49	1·33
KCN + phosphate	0·87	1·43	1·30

When the organisms were placed in boiling water for 5 min. the initial O.D. value was increased (Table 4). Addition of NaCl caused an immediate decrease followed by a slower decrease to that of the control (unboiled) O.D. value. Despite the denaturation of the proteins, changes in organism size are possible when sodium ions and water enter it. Lysis appears to play little part in this effect. It could not explain the rapid and slow components of the curve nor the fact that the change stopped when the control O.D. value was reached. When sucrose was used instead of NaCl, a smaller decrease in O.D. occurred, indicating that the damaged membrane still presented a partial barrier to sucrose. The effect of heating the organisms to 50° for 15 min. shows the difference between the effects of NaCl and sucrose more clearly (Table 4). The initial O.D. value was again increased. The effect of NaCl was minimal, but that of sucrose was only slightly less than it was on normal organisms.

The effect of freezing and thawing was somewhat different from that of heat (Table 4). The initial O.D. was not affected, but NaCl was unable to increase the O.D., which decreased progressively with time to 60–70% of the initial value;

i.e. the organism had become much larger or some had been lysed. Sucrose, however, was still able to increase the O.D. and this value did not decline below that of the initial.

Table 4. *The effect of heat and of freezing and thawing suspensions of* Pseudomonas aeruginosa *on the change in optical density* (O.D.) *value produced by 0·1 M-NaCl or 0·2 M-sucrose*

		Time of observation				
		Initial	Zero time	60 min.	120 min.	180 min.
		O.D. value				
Pre-treatment	Agent					
None	NaCl	1·10	1·82	1·71	1·53	1·43
Boiled 5 min.	NaCl	1·65	1·26	1·16	1·10	1·00
None	Sucrose	1·08	1·71	1·43	1·43	1·43
Boiled 5 min.	Sucrose	1·65	1·55	1·48	1·43	1·43
None	NaCl	1·18	1·96	1·72	1·55	1·43
At 50° for 15 min.	NaCl	1·44	1·60	0·90	0·83	0·82
None	Sucrose	1·18	1·93	1·55	1·43	1·40
At 50° for 15 min.	Sucrose	1·44	1·82	1·60	1·55	1·43
Freezing and thawing	NaCl	1·09	0·86	0·63	0·58	—
	Sucrose	1·09	1·45	1·09	1·09	—

The effect of organic anions

Sodium succinate was compared to sodium chloride as the osmotic agent. The increase of O.D. value with NaCl at zero time was 0·86 and at 60 min. it was 0·73 units; with sodium succinate the respective figures were 0·73 and 0·27 units. Similar results were obtained with the sodium salts, sodium pyruvate, formate, acetate, benzoate and salicylate. The organic anions, whether possible sources of energy or not, caused a rapid decrease in O.D. to the initial value.

The effect of EDTA, benzalkonium chloride and sodium alkylbenzene sulphonate

These compounds were added to the organism suspension 15 min. before dilution and addition of NaCl or sucrose. EDTA (ethylenediaminetetra-acetic acid) and benzalkonium chloride increased the initial O.D.; Na alkylbenzene sulphonate had no effect on it. EDTA partially inhibited the rise in O.D. with NaCl, and the decrease in O.D. with time was more rapid. EDTA had no effect on the action of sucrose. After benzalkonium chloride, NaCl caused only a decrease in O.D. as if the barrier to its entry were eliminated, but the response to sucrose was little affected. After alkylbenzene sulphonate, NaCl produced a normal increase in O.D. but the subsequent decrease in O.D. was more rapid than usual. At the concentration used this anionic detergent did not prevent the initial effect of NaCl but did allow the subsequent more rapid entry of the salt into the organisms. Again the action of sucrose was not affected. These results are shown in Table 5.

All the effects of sucrose listed above were observed with glucose. The effects of KCl were the same as those of NaCl except that the decrease in O.D. with time was more rapid.

Experiments were done with a strain of *Escherichia coli* obtained from the clinical laboratory and the differences between it and the *Pseudomonas aeruginosa* can be

summarized as follows. Sodium chloride increased the O.D. but the percentage increase was 15% smaller than that produced in *P. aeruginosa*. Osmolar sucrose had only a slight transient effect. Apparently *E. coli* is readily permeable to sucrose. Glucose increased the O.D. with *E. coli* to the same extent as did NaCl. Sodium succinate and pyruvate were as effective as NaCl but sodium formate, acetate and benzoate were inactive. Heating to 50° and freezing and thawing were without effect, but after boiling, *E. coli* behaved like *P. aeruginosa*. EDTA and alkylbenzene sulphonate in the concentrations used, had no effect but benzalkonium chloride was active.

Table 5. *The effect of pre-incubation of suspensions of* Pseudomonas aeruginosa *with EDTA, benzalkonium chloride (BA) and sodium alkylbenzene sulphonate (AS) on the change in optical density* (O.D.) *values produced by 0·1 M-NaCl or sucrose*

In these experiments the osmolarity of sucrose was half that of NaCl.

		Time of observation			
		Initial	Zero time	60 min.	120 min.
		O.D. value			
Pre-treatment	Agent				
None	NaCl	1·08	2·12	2·01	1·89
EDTA 50 μg.	NaCl	1·26	1·72	1·36	1·26
None	Sucrose	1·08	1·87	1·65	1·54
EDTA 50 μg.	Sucrose	1·26	1·89	1·65	1·62
None	NaCl	0·73	1·35	1·18	1·17
BA 50 μg.	NaCl	0·95	0·68	0·53	0·53
None	Sucrose	0·73	1·25	1·09	1·00
BA 50 μg.	Sucrose	0·99	1·34	0·95	0·93
None	NaCl	1·26	1·95	1·72	1·64
AS 120 μg.	NaCl	1·26	1·95	1·35	1·18
None	Sucrose	1·26	2·01	1·82	1·72
AS 120 μg.	Sucrose	1·26	2·01	1·78	1·72

DISCUSSION

The osmotic effect produced by the various compounds on these bacteria depends on the characteristics of the cell membrane, i.e. its ability to impose a barrier to free diffusion. Urea, for instance, diffuses through most membranes and causes no change in O.D. when added to suspensions of these organisms. The results with this simple method show some interesting differences in membrane characteristics. For instance, *Pseudomonas aeruginosa* has a barrier to sucrose, *Escherichia coli* has not. Heat, and freezing and thawing destroy the barrier to cations but not to sucrose in *P. aeruginosa*. Similar treatment had no effect on the ion barrier in *E. coli*. *P. aeruginosa* is more sensitive to the action of surface-active agents than *E. coli*, and in the former organism these agents affect the barrier to cations at concentrations which do not affect the barrier to sucrose. When the organisms *P. aeruginosa* and *E. coli* were allowed to take up cations before being subjected to the osmotic shock the change in O.D. was smaller. Another factor which would impose a limit to change in size of organism is the elasticity of the cell wall; this might explain why an increase in osmolarity above the equivalent of 0·1 M-NaCl had little or no effect. The rate of decrease in O.D. following the initial increase seems to depend on

several factors. First, the rate of cation uptake. When NaCl was used as the osmotic agent, the mean decrease in O.D. in 60 min. was 0·20; when KCl was used it was 0·38 units. Secondly, the nature of the anion. The rates of decrease of O.D. with NaCl, NaBr and $NaNO_3$ were the same. But when the anion was organic the rate of decrease was consistently more rapid. There is no obvious explanation for this. Finally, pre-treatment of *P. aeruginosa* with EDTA or benzalkonium chloride increased the initial O.D. This implies that these agents allowed an outward diffusion of ions accompanied by water, with the consequent shrinking of the organisms.

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EXPLANATION OF PLATE 1

Fig. 1. Electron-microscope picture of washed *Pseudomonas aeruginosa* organisms after suspension in water × 16,800.

Fig. 2. After suspension in 0·15 M-NaCl × 16,800.

The pictures were taken by Dr J. R. Overman of the Department of Microbiology on an RCA–EML electron microscope.

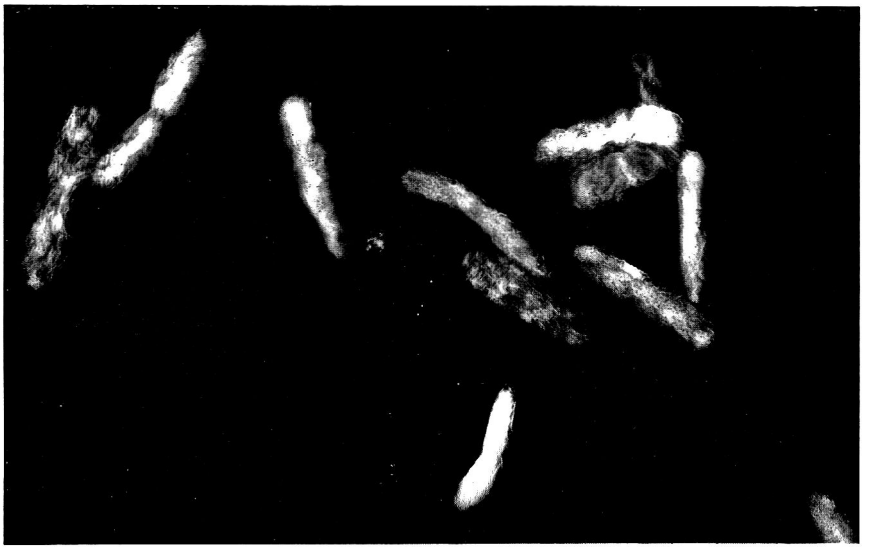

Fig. 1

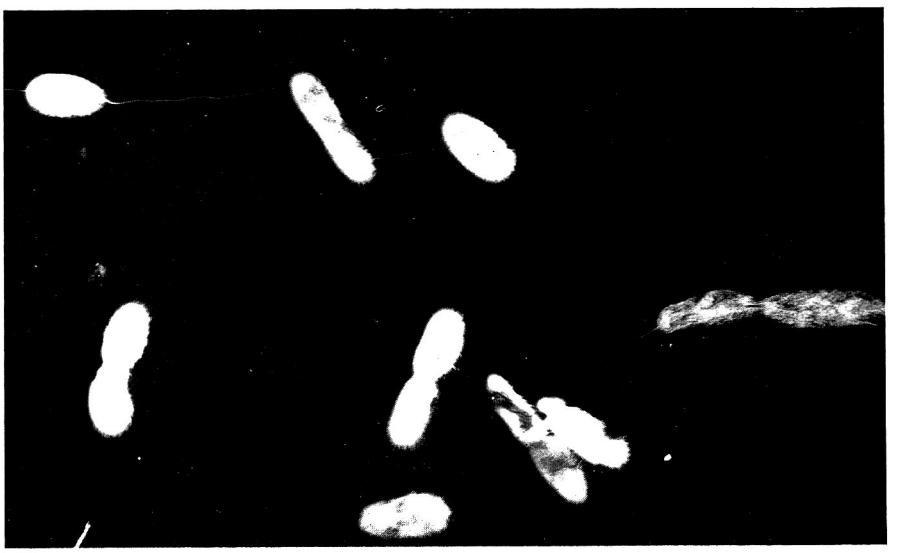

Fig. 2

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Structure and Replication of the Trachoma Agent in Cell Cultures, as shown by Electron Microscopy

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(*Received* 25 *April* 1962)

SUMMARY

Propagation of the trachoma agent in human cell cultures has made possible the observation by electron microscopy of sequential stages in the replication of the micro-organism. Scattered reticular foci with incomplete limiting membranes were present in the cytoplasm of HeLa cells 12 hr. after exposure to the infectious agent in high multiplicity. After 24 hr. the foci had coalesced to give a single reticular mass. Within it there appeared large and discrete developmental forms from which, by progressive subdivision and diminution in size, typical elementary bodies were formed. The elementary bodies had a prominent limiting membrane, and a dense core within which a fibrillar nucleoid could be recognized. The controversial taxonomic status of the trachoma agent is considered briefly in the light of ultrastructural evidence.

INTRODUCTION

Members of the psittacosis group, with the related causal agents of trachoma and inclusion blennorrhoea, present an interesting and controversial problem in taxonomy (Andrewes, 1952; Bedson, 1959). Although acknowledged as obligate intracellular parasites and virus-like in size, the micro-organisms have properties that prompt misgivings about their viral nature, for they appear to have much in common with the bacteria and rickettsias. There is increasing support for the view expressed by Weiss (1955) that they are, in fact, typical of neither rickettsias nor viruses but lie somewhere between the two. The features usually cited as incompatible with a viral classification are: (i) microscopical studies have consistently upheld the view that multiplication is by binary fission of intact particles; (ii) chemical analyses indicate that the infective particles have a chemical complexity akin to that of bacteria; (iii) the occurrence of an eclipse phase is unproven; (iv) the organisms are susceptible to a variety of antibiotics and other compounds. The issue is by no means settled, however, and some recent investigations draw attention to the virus-like aspects of the psittacosis group. McCloskey & Morgan (1961) observed that the latent phase following inoculation of L cell cultures with psittacosis virus could be prolonged for 24 hr. by withholding certain vitamins and amino acids: during this period infectivity was lost, and no viral structures or inclusion bodies were detected by light or electron microscopy. Infectious virus reappeared in the system within 24 hr. of rectifying the nutritional deficiency. Tanami, Pollard & Starr (1961) studied the effects of pyrimidine analogues and *p*-fluorophenylalanine on the growth curve of psittacosis virus, also in tissue cultures. It was inferred that a lag of 10 hr.

occurred between viral DNA synthesis and its incorporation into infectious particles, and it was suggested that replication of 'naked' DNA indicates an essentially viral mode of multiplication. In similar vein Becker, Mashiah & Bernkopf (1962), describing a growth cycle of some 72 hr. for the trachoma agent *in vitro*, reported that it passed initially through a non-infectious phase lasting 24 hr.

Following isolation of the trachoma agent in the yolk sac of chick embryos (T'ang, Chang, Huang & Wang, 1957) it was propagated successfully in epithelial cell cultures, producing a widespread cytopathic effect typical of the psittacosis group (Furness, Graham, Reeve & Collier, 1960; Bernkopf, Mashiah & Maythar, 1960). Electron microscopical study of such a system seemed an opportune means of gaining more precise information, in morphological terms, about multiplication of the trachoma agent, and so perhaps of the psittacosis group as a whole.

METHODS

Cell cultures. Stock cultures of HeLa cells in 250 ml. bottles were grown in a medium consisting of Gey's balanced salt solution, 10% (v/v) human serum inactivated at 56° for 30 min. and 0·5% (w/v) lactalbumin hydrolysate, with streptomycin 200 μg./ml. Cells were subcultured into half test tubes containing 7 mm × 22 mm. coverslips, using the same medium but without antibiotics.

Inoculum. The T'ang TE55 strain of the trachoma agent (T'ang *et al.* 1957), originally supplied to us by Dr L. H. Collier (The Lister Institute) was maintained in this laboratory by serial yolk-sac propagation in chick embryos (Collier & Sowa, 1958). Infected yolk-sac tissue was ground in a tissue grinder with 0·85% saline containing 1% (v/v) horse serum and 10% (v/v) nutrient broth, centrifuged at 2500 rev./min. for 10 min., and the supernatant fluid stored in sealed glass capillaries at −70°.

Assay and inoculation. The infectious material was titrated in HeLa cells by a method essentially similar to that of Furness, Graham & Reeve (1960). An inoculum capable of infecting 90–100% of the cells was prepared by 1/100 dilution of the yolk-sac material in Gey's solution; 0·5 ml. of inoculum in each tube was left to adsorb for 1 hr. at 37°. It was then removed, fresh antibiotic-free medium added, and incubation continued at 37°.

Electron microscopy. Infected cultures were processed for electron microscopy, together with uninoculated controls, at intervals of 6, 12, 24, 48, and 72 hr. after inoculation. The samples for each time consisted of pooled cells from batches of six tubes. The culture medium was first removed, and replaced with 3 ml. of the 1% (w/v) osmium tetroxide fixative of Palade (1952) at pH 7·4; after a few minutes the cells were pushed gently from the tube walls into the fixative by using a rubber tipped glass rod. The contents of the six tubes were mixed and fixation continued for 1 hr. at room temperature. In loose pellet form the cells were then washed, dehydrated, and infiltrated with methacrylate monomer in the usual way. Final embedding was in a prepolymerized 1+4 mixture of methyl and *n*-butyl methacrylates, catalysed with 1% (w/v) benzoyl peroxide. Blocks of suitable hardness resulted from standing at 60° for 24 hr.

Sections were cut with a Porter–Blum microtome and collected on copper grids bearing carbon-stabilized nitrocellulose films. Before examination most of the

grids were floated face downwards for 3 hr. on a solution of 5 % (w/v) uranyl acetate in 1 % (v/v) acetic acid (Valentine, 1962), washed vigorously for about 30 sec. in distilled water and allowed to dry. The lead hydroxide method of Dalton & Zeigel (1960) was also used. The sections were viewed with a Siemens UM 100 electron microscope operating at 60 kV; pictures were taken at ×1250 or ×7000 on ultrafine grain Ilford N.60 (photomechanical) plates, and subsequently enlarged.

Light microscopy. The progress of cytopathic change in the cultures was assessed, before electron microscopy, by inspection of the coverslips placed for this purpose into the culture tubes. Coverslips from each of the inoculated batches, and from uninfected control tubes, were fixed for about 20 min. in Palade's buffered osmium tetroxide solution; after rinsing in Gey's solution they were transferred to 70 % (v/v) ethanol in water for about 24 hr. They were then mounted in water, wax-sealed and examined by phase-contrast microscopy as described elsewhere (Armstrong & Pereira, 1960). Similar monolayers were stained by the periodic acid-Schiff (PAS) method for demonstration of polysaccharides, and counterstained with haematoxylin. Identification of glycogen was confirmed by treatment of some cultures with saliva for 45 min. at 37°, before PAS staining. A few coverslip cultures were ethanol-fixed for Giemsa staining.

RESULTS

Phase-contrast microscopy

Inspection of the osmium-fixed cell monolayers revealed a progression of cellular changes after inoculation. Those examined after 6 hr. were not visibly different from the control HeLa cells. After 12 hr. a small optically dense nodular mass was detectable to one side of the nucleus in many cells, and by 24 hr. a well-defined juxtanuclear inclusion was present in almost every cell. It had a vesicular mottled appearance and continued to enlarge until 72 hr. after inoculation, when the experiment was terminated. The cells were then grossly distended, in each the nucleus was displaced to the periphery by a very large vesicular inclusion containing aggregates of dense particulate material (Pl. 1, fig. 1). These findings are in line with recent descriptions of trachoma-infected tissue cultures (Gordon, Quan & Trimmer, 1960; Furness, Graham, Reeve & Collier, 1960; Bernkopf *et al.* 1960), and comparable with the earlier accounts of changes that accompany multiplication of other members of the psittacosis group (Bedson & Bland, 1932). The cytopathic changes developed synchronously throughout the cell sheets; counts indicated that more than 90 % of the cells were infected at the outset. At 72 hr. the presence of a few cells with 'early-type' inclusions suggested the onset of a second cycle of growth.

Electron microscopy

The elementary body. Observations were directed first towards the contents of the fully developed inclusion or vacuole, in cells fixed 72 hr. after inoculation. The object was to determine the structural features of the infectious trachoma elementary bodies, believed to occur in the inclusion at this late stage, before attempting to interpret changes leading to their formation. There was no difficulty in finding inclusions in the sectioned cells. They contained a large number of small, rounded or oval particles of high electron density dispersed within a matrix of very much

lower density. Measurements gave an average particle size of 375 × 265 mμ, mean diameter about 320 mμ. The long axes of those presenting an oval profile were invariably orientated in one direction, suggesting that truly spherical structures had undergone the usual compression on sectioning. When allowance is made for differences due to the plane of section, the regularity in size and form of the dense particles was such as would be expected of viral elementary bodies.

Part of a typical 72 hr. inclusion is illustrated at high magnification in Pl. 1, fig. 2; the area shown contains four of the objects which we regard as elementary bodies. Each had a prominent and dense inner core (mean diameter 220 mμ) separated by a clear zone from a characteristically folded limiting membrane. The thickness of the membrane was about 10 mμ, but often looked more in the plane of a section because of its folded nature. This interpretation of cross-sections is entirely consistent with the 'wrinkled pea' description of intact elementary bodies, as revealed by electron microscopy of air-dried and metal-shadowed material from trachoma-infected chick embryos (Collier, 1959). The inner cores seemed on section to be of two kinds. One was a composite mass of fibrillar and granular elements, forming a compact structure with a regular outline. The other kind was less regular in outline and had the fibrillar component segregated from the granular part by a distinct but usually incomplete membrane. We shall refer to this saccular structure, which measured some 130 mμ in diameter and was of great electron density, as a nucleoid (Pl. 1, figs. 2, 3). Inspection of random thin sections cannot establish whether an organized nucleoid is, in fact, entirely lacking in cores of the first kind; but it is conceivable that the variations in core structure signify different levels of particle maturity.

Appearance 6 hr. after inoculation. Many of the cells examined at this stage were not obviously different from those of the uninoculated control cultures. However, two abnormal features appeared quite often in the sections and were undoubtedly a consequence of the preceding inoculation. Pale mottled areas occurred in the cytoplasm ranging in size from small patches as seen in Pl. 2, fig. 4, to confluent masses larger than the cell nucleus. At high magnification they appeared to be largely amorphous but with a variable content of moderately dense 15–30 mμ particles, closely comparable with the large type of glycogen deposits observed in liver cells after osmium fixation (Revel, Napolitano & Fawcett, 1960). As a test of this interpretation, sections were floated on a solution of potassium permanganate as advocated by Drochmans (1960); the previously pale areas developed great electron density and became the most conspicuous objects in the cell cytoplasm. Further confirmation came from examination with the light microscope of PAS-stained monolayer cultures; almost all cells were seen to contain one or more large masses of PAS-positive material which was absent after saliva digestion. Negligible amounts of glycogen were demonstrable in the control cultures.

The other abnormality was a heterogeneous collection of small dense bodies in a juxtanuclear position, usually intermingled with the Golgi complex. Occasional structures of the same kind occurred in cells of the uninoculated cultures. A typical cluster within a cell inoculated 6 hr. previously is shown in Pl. 2, fig. 4. The profiles average some 350 mμ in size, variation being attributable at least in part to plane of section. Until quite recently structures of this kind have been referred to in electron microscopical investigations in general terms, e.g. 'dense body', 'micro-

body', 'lamellar body' and so on. Studies on mammalian liver cell structure (Essner & Novikoff, 1960; Daems & van Rijssel, 1961) have established the identity of peribiliary dense bodies as lysosomes, i.e. membrane-limited organelles containing acid phosphatase and other enzymes, which are directly concerned in phagocytic and lytic activities of the cell (De Duve *et al.* 1955). The morphological heterogeneity of lysosomes has been emphasized, but typically they contain ferritin-like granules and have a single limiting membrane. Many of the dense bodies with which we are presently concerned conformed to the descriptions of liver cell lysosomes and their presence would be consistent with recent phagocytic activity. Amongst them, however, was an occasional example having a dense fibrillo-granular central area surrounded by a markedly folded membrane. There was a superficial similarity between such forms and trachoma elementary bodies. Nevertheless, close examination of many cells fixed 6 hr. after inoculation did not reveal structures, in the cytoplasm or at the cell surfaces, that could be identified unequivocally as elementary bodies or their derivatives.

Appearance 12 hr. after inoculation. Some degree of alteration was now detectable in almost every cell examined. Lysosome aggregates were less obvious than at 6 hr., but large glycogen deposits were much in evidence. Light microscopy of PAS and haematoxylin-stained monolayers at this stage revealed, in addition to cytoplasmic glycogen, groups of basophilic particles in the general vicinity of the Golgi complex. They had the typical appearance of early developmental forms of the psittacosis group (Bedson & Bland, 1932).

The first indication of structural elements presaging specific cytoplasmic inclusions was given in micrographs at this stage. At lower magnifications it was possible to detect in the cytoplasm more or less rounded formations, distinct from the glycogen and unlike any normal component of the HeLa cell. Ranging in size from under 1μ to more than 2μ in diameter, they had on section the consistency of a fine reticulum of low to moderate electron density. Several such reticular foci are depicted within a limited area of the cell cytoplasm in Pl. 3, fig. 5. Although sometimes grouped in this way, individual foci were also more widely scattered in the cells. At higher magnification each reticular focus appeared to consist essentially of a meshwork of delicate and wavy fibrils, usually bounded by a well-defined bilaminar membrane. In some, however, notably those over $1{\cdot}5\mu$ in diameter, the enclosing membrane was fragmentary or incomplete, the fibrillar contents being then in direct continuity with surrounding cytoplasm. Similar fibrillar material was sometimes observed without a vestige of a limiting membrane. No structures resembling elementary bodies were found.

Appearance 24 hr. after inoculation. There was no difficulty after 24 hr. in locating with the electron microscope characteristic juxtanuclear inclusions, corresponding to those observed by phase-contrast in the osmium-fixed monolayers. Indeed, they were of such size that at least part of one was encountered in almost every cell sectioned. The inclusion appeared as a localized area of altered cytoplasm several microns in diameter, with no equivalent in the uninoculated control cells. The presence of the abnormal structure caused slight indentation of the otherwise normal cell nucleus, and a corresponding bulge was often visible in the cell wall overlying the inclusion. Mitochondria, ribosomes and endoplasmic reticulum were absent from the area occupied by the inclusion, and abnormal glycogen deposits

were distinctly smaller and less numerous than in the specimens fixed at 6 hr. and 12 hr. after inoculation.

The fine structure of the inclusion was remarkably constant from cell to cell. It consisted for the most part of a fibrillar matrix or reticulum of moderate overall density, incorporating somewhat denser patches each partially enclosed by an incomplete and fragmentary bilaminar membrane. The character of the inclusions suggested that the separate reticular foci seen at 12 hr. had become more numerous and were forming a confluent mass (Pl. 4, fig. 6). It was a regular finding that no membrane or other definable barrier separated the interior of the inclusion from the surrounding cell cytoplasm. Detail from a typical inclusion is shown at higher magnification in Pl. 5, fig. 7: curved profiles of several incomplete bilaminar membranes are present, and the components of the reticulum are seen as wavy fibrils, of high density and about 4 mμ in thickness. Their length could not be determined from the study of sections. No elementary bodies were seen in the cells at this time.

Appearance 48 and 72 hr. after inoculation. A remarkable change in the character of the intracellular inclusions took place in the interval between 24 and 48 hr. after inoculation, accompanied by a considerable distortion of host-cell morphology. As anticipated from the preliminary phase-contrast observations there was pronounced enlargement of the inclusion, which now appeared as a distinctly vesicular formation with a diameter of 20μ or more. The cell nucleus was displaced to the side, markedly compressed by the inclusion but otherwise structurally unaltered. Recognizable cytoplasmic elements were restricted to a perinuclear area and a tenuous peripheral rim around the massive inclusion. Degenerative changes in the mitochondria were conspicuous in some cells. Little remained of the glycogen deposits seen at early stages.

As in the earlier stages, examination at high magnification revealed no continuous morphological barrier between the interior of the inclusion and the surrounding cytoplasm. The boundary is therefore probably a phase interface rather than a morphological entity. Each inclusion contained a number of discrete, more or less round bodies suspended within a matrix of comparatively low density (Pl. 5, fig. 8). The bodies were remarkably variable in size and structural detail, but all were alike in possessing a complete and well-defined limiting membrane; they will be referred to as developmental forms of the trachoma agent. The largest measured 3 or 4μ in diameter and the smallest between 300 and 400 mμ; different sizes occurred together in the majority of inclusions, but occasional cells appeared to contain only the larger ones. The larger developmental forms contained a reticulum of fibrillar nature similar to that observed, in an unenclosed form, in cultures examined at the 24 hr. stage. In addition, many of the larger forms also contained a number of prominent condensations of fibrillar material, round or oval in shape and about 300 mμ in diameter (Pl. 5, fig. 8). Complication of the cross-sectional morphology of the large forms frequently arose from the occurrence of deep finger-like invaginations of the limiting membrane, producing profiles of a bizarre nature. Inspection of developmental forms of decreasing size revealed a progression towards profiles of a more regular character, with increasing density and more even distribution of contents. Within forms typical of the intermediate size range (say, 0·5–1μ in diameter) one or two centrally placed fibrillar condensations were separated from

the limiting membrane by a wide zone occupied by material of a different nature, predominantly granular and of comparatively low electron density (Pl. 6, fig. 9). Developmental forms of smaller size than this became virtually indistinguishable from elementary bodies.

The cultures fixed 72 hr. after inoculation contained many cells similar to those seen at 48 hr.; but in general the inclusions were even larger and host-cell components correspondingly more displaced. Inside the inclusions elementary bodies predominated, with a reduced number of developmental forms of the large and intermediate sizes (Pl. 7, fig. 12; Pl. 8, fig. 13). In addition, a few disrupted large forms and some others that appeared to be intact but devoid of contents were often visible amongst the elementary bodies.

A considerable part of each inclusion was accounted for by the matrix in which developmental forms and elementary bodies were suspended. Owing to low overall density there was little to indicate its composition, except for scattered particles of moderate density, 20–50 mμ in size, sometimes aggregated to form larger stellate clusters (Pl. 8, fig. 14). This is reminiscent of descriptions of particulate glycogen (Revel *et al.* 1960). This identification was borne out by the PAS-stained monolayer cultures; the contents of inclusion bodies were strongly PAS-positive from the 48 hr. stage.

Evidence of fission. Arbitrary classification of the developmental forms on a basis of size or structural differences cannot be justified; a whole range of transitional forms was recognizable in the electron micrographs, from the largest down to the elementary bodies. From comparison of the contents of cytoplasmic inclusions in 48 and 72 hr. specimens, it could be inferred that the larger developmental forms were somehow being replaced by those of smaller size. A search was therefore made for profiles that might be a key to inter-relationship of the various forms, bearing in mind the many previous reports of binary fission in the psittacosis group. In the 48 hr. inclusions there were always a few developmental forms so shaped, or disposed in relation to each other, as to suggest they had indeed been dividing at the time of fixation. Furthermore, it seemed that at least two modes of division could be recognized, namely into unequal or equal parts. Unequal division was shown mainly by the largest forms, and could be described as budding. Examples of it are illustrated in Pl. 6, fig. 10: here, two forms just a little larger than elementary bodies are each situated in close apposition to a comparatively large form, from which it could be supposed they were separating when fixed. The frequency of such associations made it unlikely they were entirely fortuitous; especially as large forms were also to be found showing localized protrusions which, though still continuous with the membrane of the parent body, exhibited varying degrees of intervening constriction. On the other hand, many intimately paired profiles of smaller developmental forms seemed to suggest division into equal halves (Pl. 6, fig. 11; Pl. 8, fig. 13). In general, the smaller the forms concerned, the more closely did their division approximate to binary fission as the term is generally understood. There were frequent signs of such divisions in the inclusions at 72 hr., when the larger developmental forms had mostly disappeared; and the possibility of elementary bodies undergoing repeated binary fission cannot be ruled out.

DISCUSSION

The first systematic observations on the growth of psittacosis virus in infected tissues and cell cultures were based on conventional staining techniques for light microscopy (Bedson & Bland, 1932, 1934; Bland & Canti, 1935). Similar work was undertaken on the closely related causal agents of lymphogranuloma venereum and meningopneumonitis (Findlay, Mackenzie & MacCallum, 1938; Rake & Jones, 1942; Higashi, Notake & Fukada, 1959). These researches have led to a wide measure of agreement about the cytopathic changes that accompany multiplication of the psittacosis group of organisms, the process culminating in a mass of newly formed elementary bodies less than 0·5μ in size. It has been generally considered that invading particles retain their integrity in the host cell cytoplasm and become transformed, after a latent period of 5 to 20 hr. into a visible cluster of comparatively large structures known as initial bodies, between 1 and 2μ in size. These seem to undergo repeated binary fission which, with a decrease in size, gives origin to the new infectious particles. The term 'plaque' was applied to large cytoplasmic structures of homogeneous appearance. Although plaques were first interpreted as plasmodia of viral nature (Bedson & Bland, 1932) this view was later rejected in favour of regarding them as just closely packed colonies of initial bodies (Bedson & Bland, 1934; Bedson, 1959). Light microscopy of cell cultures inoculated with the trachoma agent revealed an almost identical sequence of cytopathic changes (Gordon *et al.* 1960; Furness *et al.* 1960; Bernkopf *et al.* 1960). In this connexion it is interesting to recall that a similar developmental cycle was postulated many years ago after examination of cell inclusions in tissue from trachoma cases (Halberstaedter & von Prowazek, 1907; Lindner, 1910).

Although light microscopy clearly demonstrates affinities within the psittacosis group, limited resolving power has impeded the understanding of early stages in the growth cycle. The uncertain nature of markedly pleomorphic developmental forms, and doubts concerning the degree of association between the infectious agent and the host cell, have been obstacles in the way of comparing satisfactorily the reproductive mechanisms of the psittacosis group and other micro-organisms such as the bacteria (Weiss, 1955; Trager, 1960). Already in several laboratories workers have turned to electron microscopy in the hope that greater resolving power would be decisive in the vexed question of taxonomic relationships. Thin-section studies have been made of cells infected with the agents of meningopneumonitis (Gaylord, 1954; Tajima, Nomura & Kubota, 1957; Higashi, 1959), feline pneumonitis (Litwin, 1959), psittacosis (Litwin, Officer, Brown & Moulder, 1961) and trachoma (Mitsui *et al.* 1958). Materials examined included tissues of infected animals and embryonated eggs, as well as inoculated cell cultures; but in terms of advancing previous knowledge the outcome has been inconclusive. Selective viewing of fragments from an organized tissue system is not an ideal basis for the analysis of a complex developmental cycle; and most of the published accounts deal chiefly with the structure of well-developed cytoplasmic inclusions. As pointed out by Higashi (1959) morphological changes giving origin to the large developmental forms have proved elusive; also, the internal structure of the elementary bodies has not so far been described in detail.

The findings of Tajima *et al.* (1957) in a study of chick chorioallantoic membranes,

fixed at intervals from 2 hr. to 4 days after inoculation with the virus of meningopneumonitis, are of exceptional interest. For this group of workers reported a localized 'viral matrix' in the cytoplasm of infected cells, before the appearance of discrete developmental forms with limiting membranes. The matrix was described as granular, not sharply demarcated from the surrounding cell cytoplasm, and similar to the diffuse viroplasm characterizing the initial stages of intracellular multiplication of pox viruses. Nevertheless, Litwin *et al.* (1961) were unable to

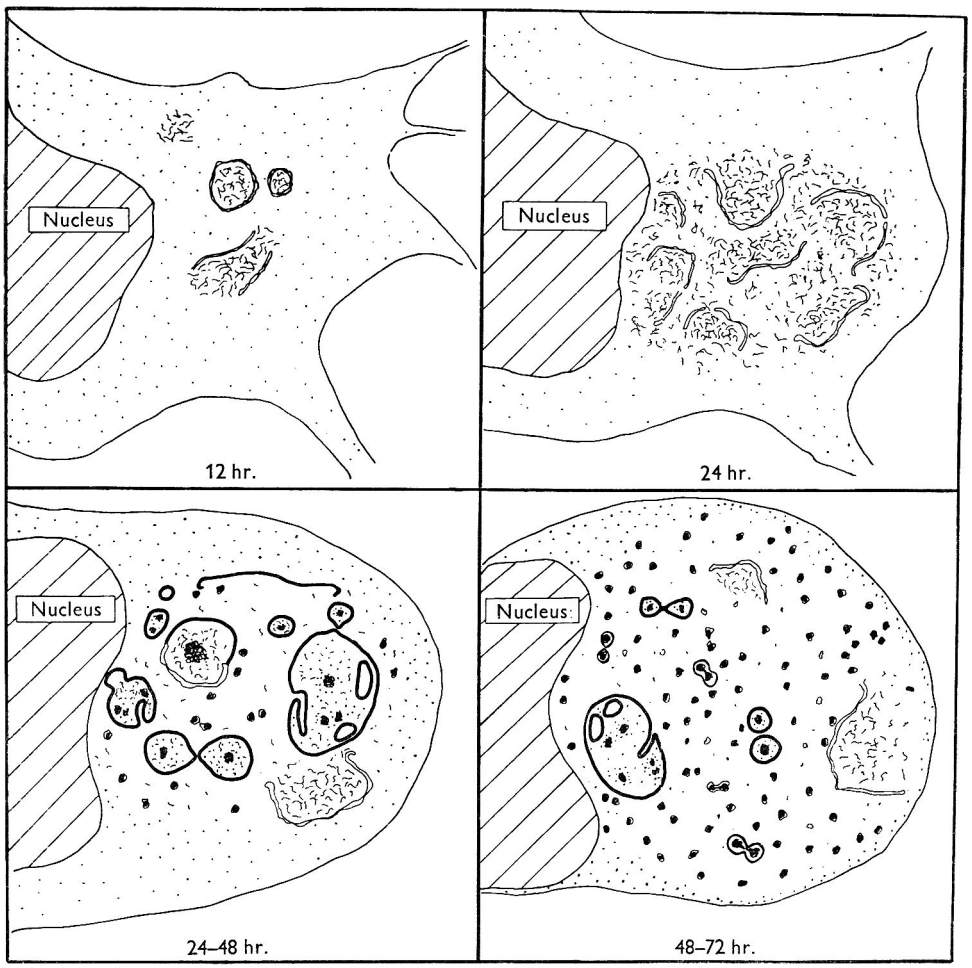

Fig. 1. Representation of stages in the developmental cycle of the trachoma agent in HeLa cells.

identify a similar matrix in psittacosis-infected chorioallantoic membranes; they concluded that a mechanism involving such elements 'cannot be an important means of multiplication in the psittacosis group'.

In the present investigation a regular sequence of changes was observed in HeLa cells inoculated with a high multiplicity of the trachoma agent. It has been possible from this to reconstruct some important aspects of the complex cycle of intra-

cellular growth. Four significant stages are shown schematically in Fig. 1. The first was seen 12 hr. after inoculation, and is the *stage of scattered reticular foci*, with incomplete limiting membranes. Coalescence of these between 12 and 24 hr. produces the single inclusion of the *stage of confluent reticulum*. There can be little doubt that the formation which characterizes this stage corresponds to the disputed 'viral matrix' described by Tajima *et al.* (1957). In the period from 24 to 48 hr. the formation of new membranes within the inclusion gives rise to a series of spherical closed compartments, with a maximum diameter of about 3μ. This is the *stage of discrete developmental forms*. Focal condensations occur within the larger forms, and between 48 and 72 hr. after the onset of infection, subdivision by means of budding and fission, with progressive diminution in size, gives rise to the final *stage of newly formed elementary bodies*. The mechanism by which newly formed elementary bodies escape from the inclusion remains obscure; transfer of individual elementary bodies through the cell wall to the exterior did not seem to be occurring in the material examined.

How infecting particles normally gain access to the host-cell cytoplasm, and their immediate fate thereafter, have not been elucidated. It is now certain that information on this could be expected only from close study of infected cells at a stage earlier than any in the present series. The demonstration recently of the phagocytosis and subsequent breakdown of vaccinia virus particles (Dales & Siminovitch, 1961) encourages the speculation that infectious particles of trachoma, and others in the psittacosis group, may be taken into the cell in this way. Certainly, the finding of conspicuous lysosome aggregates 6 hr. after inoculation in the present series suggests that a high level of phagocytic activity had been taking place. We do not know whether each of the reticular foci seen in 12 hr. specimens had developed from a single infecting particle, or had been formed *de novo* after complete disintegration of the infecting particles.

In spite of these uncertainties, points of special interest revealed by the electron microscope are the incomplete separation of the contents of early reticular foci from surrounding cytoplasm, and their subsequent fusion into a confluent mass which is the definitive inclusion of the 24 hr. stage. The possibility has to be considered that the incomplete nature of the bilaminar membranes observed in early stages of the cycle was a technical artefact, due to disruptive polymerization of the methacrylate monomer or to defective fixation. Polymerization damage has characteristic effects on cellular fine structure (Morgan, Rose & Moore, 1957) but evidence of this was encountered only in occasional blocks in this series. If polymerization damage is involved it would therefore be necessary to postulate unusual susceptibility of the early developmental forms of the trachoma agent. Fixation artefact can take various forms and is still little understood; it is therefore arguable that complete but delicate membranes may enclose the reticular foci, and be imperfectly preserved by the buffered osmium tetroxide method currently favoured by electron microscopists. In fact, it is difficult to envisage the observed fine structural details arising in this way, but we bring the view forward as one which cannot be entirely ruled out.

Correlation of electron microscopical details with the classical cytopathic changes associated with organisms of the psittacosis group seems to be largely a matter of terminology, presenting no great difficulty. Thus, the structures formerly termed

'plaques' can reasonably be equated with the confluent reticulum seen at 24 hr. in the present study. Their plasmodial nature, hitherto conjectural, is borne out by electron microscopy. The precise equivalent of 'initial bodies' is less obvious; but it is likely that both the reticular foci and the later discrete developmental forms of the present account have been included together under this single heading in earlier descriptions. With both forms of microscopy it is possible to observe the innumerable elementary bodies within mature inclusions, but their internal structure is not of course represented in the images obtained with light microscopes.

It is of special interest to consider the fine structure of trachoma-infected cells in relation to recent cytochemical studies on the growth in cell cultures of several members of the psittacosis group, including the trachoma agent itself. The acridine orange fluorescence technique, together with nuclease digestion tests, has been used to demonstrate a series of regular changes in the distribution of nucleic acids in the cytoplasmic inclusions; the results were correlated with infectivity studies (Starr, Pollard, Tanami & Moore, 1960; Pollard, Starr, Tanami & Moore, 1960; Becker *et al.* 1962). During the first 24 hr., corresponding to the stages referred to here as scattered reticular foci and confluent reticulum, one or more small bodies became visible in the cytoplasm and then enlarged into a single juxtanuclear mass; this gave a strong red fluorescence signifying the accumulation of newly synthesized ribonucleic acid (RNA). As the mass enlarged further its red fluorescence tended to diminish and increasingly numerous points of intense yellow-green fluorescence emerged, indicating the development of particles containing deoxyribonucleic acid (DNA) within the preformed pool of RNA-containing material. This coincided with a rise of demonstrable infectivity. It was at about this stage in the present experiments, that discrete developmental forms were first seen in the micrographs. The presence of DNA may well be related to the more or less organized fibrillar component revealed in the elementary bodies by electron microscopy. Indeed, the ultrastructural complexity of the elementary bodies is consistent with the findings of chemical analysis which suggest that micro-organisms of the psittacosis group contain not only DNA, but also RNA and muramic acid (Zahler & Moulder, 1953; Ross & Gogolak, 1957; Allison & Burke, 1962). A glycogen-containing matrix inside the fully developed cytoplasmic inclusions of trachoma-infected cells has been known for some years (Rice, 1936; Thygeson, 1938) and is merely confirmed by the present observations. However, the formation of widespread glycogen deposits in the host cell cytoplasm, as early as 6 hr. after inoculation, does not seem to have been noted previously; the possibility of this being a response to renewal of the culture medium, rather than to infection *per se*. is suggested by recent observations on fine structural variations in normal HeLa cells (Bruni, Gey & Svotelis, 1961).

It is widely believed that the mode of intracellular replication permits a real distinction to be drawn between viral infection and parasitism by larger micro-organisms. With viruses, breakdown of invading particles results in an intimate association between the infectious agent and the host cell, initiating the synthesis of viral components from which new infectious particles are assembled; the latter do not divide. It is generally accepted on the other hand that intracellular bacteria and rickettsias remain intact, multiplying by binary fission. Difficulty in the past over classification of the psittacosis group has been attributable partly to uncertainty

concerning the degree of association between visible developmental forms and the cell, owing to limitations of the light microscope. However, complexity of the replicating mechanism for the trachoma agent is emphasized rather than diminished by electron microscopy of infected HeLa cells. The first part of the cycle is somewhat suggestive of a viral mechanism, in which derivatives of the infecting particles coalesce into a single mass of reticulum not obviously separated from the cell cytoplasm; but bacterial affinities are shown by the developmental forms which then emerge and give rise to elementary bodies through a series of divisions. In this developmental cycle the taxonomic dilemma is epitomized and it is hard to see how current concepts of viruses and bacteria allow the trachoma agent to be placed in either category. If an evolutionary process has yielded a succession of organisms decreasing in complexity from bacteria to the simplest viruses then border-line cases are only to be expected (Bedson, 1959), and their classification becomes an arbitrary question of definition. However, a theory now gaining support is that viruses and bacteria have entirely separate origins (e.g. see Allison & Burke, 1962), in which case one may reasonably expect some absolute distinction between them. Which of the several properties generally held to distinguish viruses from bacteria should be regarded as absolute, is something which can emerge only from detailed study of both groups. Only when the true criteria are recognized can the position of organisms such as the trachoma agent be settled.

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EXPLANATION OF PLATES

All figures, with the exception of Pl. 1, fig. 1, are electron micrographs of thin sections of HeLa cells from cultures inoculated with the trachoma agent.

PLATE 1

Fig. 1. HeLa cell coverslip culture 72 hr. after inoculation with the trachoma agent. In each cell there is a large vesicular inclusion and the nucleus has been pushed aside. Phase-contrast, after osmium tetroxide fixation (× 1350).

Fig. 2. High magnification of part of an inclusion in a 72 hr.-infected cell. Four sectioned elementary bodies are shown, in two of which the core is a compact fibrillo-granular structure. The other two have cores in which the fibrillar element is partially segregated within a saccular nucleoid. Note the folded limiting membranes (× 100,000).

Fig. 3. An elementary body with a particularly well differentiated oval nucleoid (× 100,000).

PLATE 2

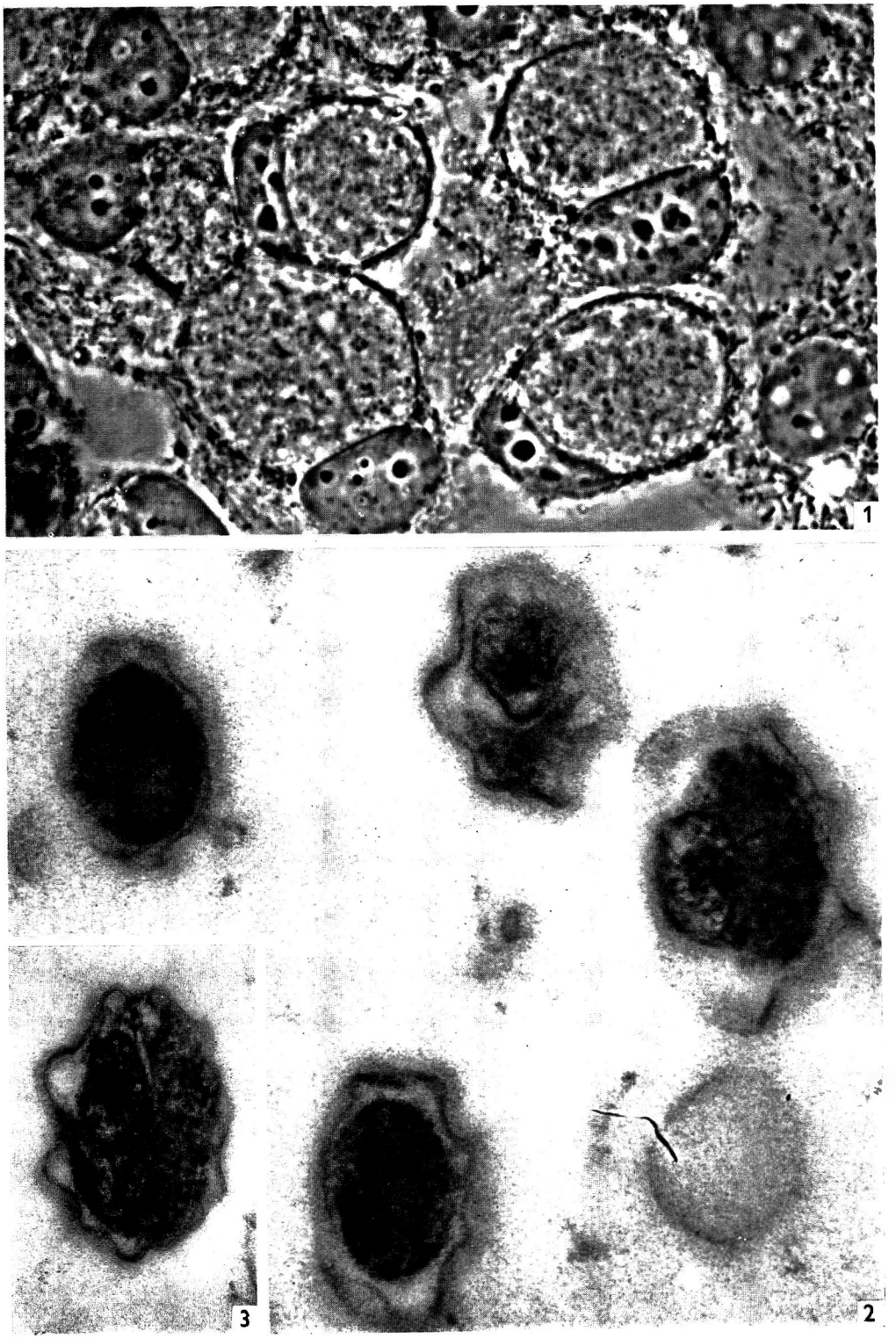
Fig. 4. Features of cytoplasmic fine structure 6 hr. after inoculation. A collection of pleomorphic lysosomes occupies a juxtanuclear position in the lower central part of the field. Low density glycogen-containing areas (Gl) were numerous and often larger than that illustrated. Normal elements include part of a cell nucleus (N), the Golgi complex (Gc) and mitochondria (M) (× 25,000).

PLATE 3

Fig. 5. Cytoplasmic reticular foci (RF), approximately 1 μ in diameter, in a cell 12 hr. after inoculation. Note the incomplete limiting membranes around some of the foci. Unidentified smaller bodies (B) are possibly precursors of reticular foci (× 23,000).

PLATE 4

Fig. 6. A localized inclusion mass between the nucleus and cell membrane of a cell from a culture inoculated 24 hr. previously. The inclusion appears to consist of coalescent reticular foci (× 20,000).



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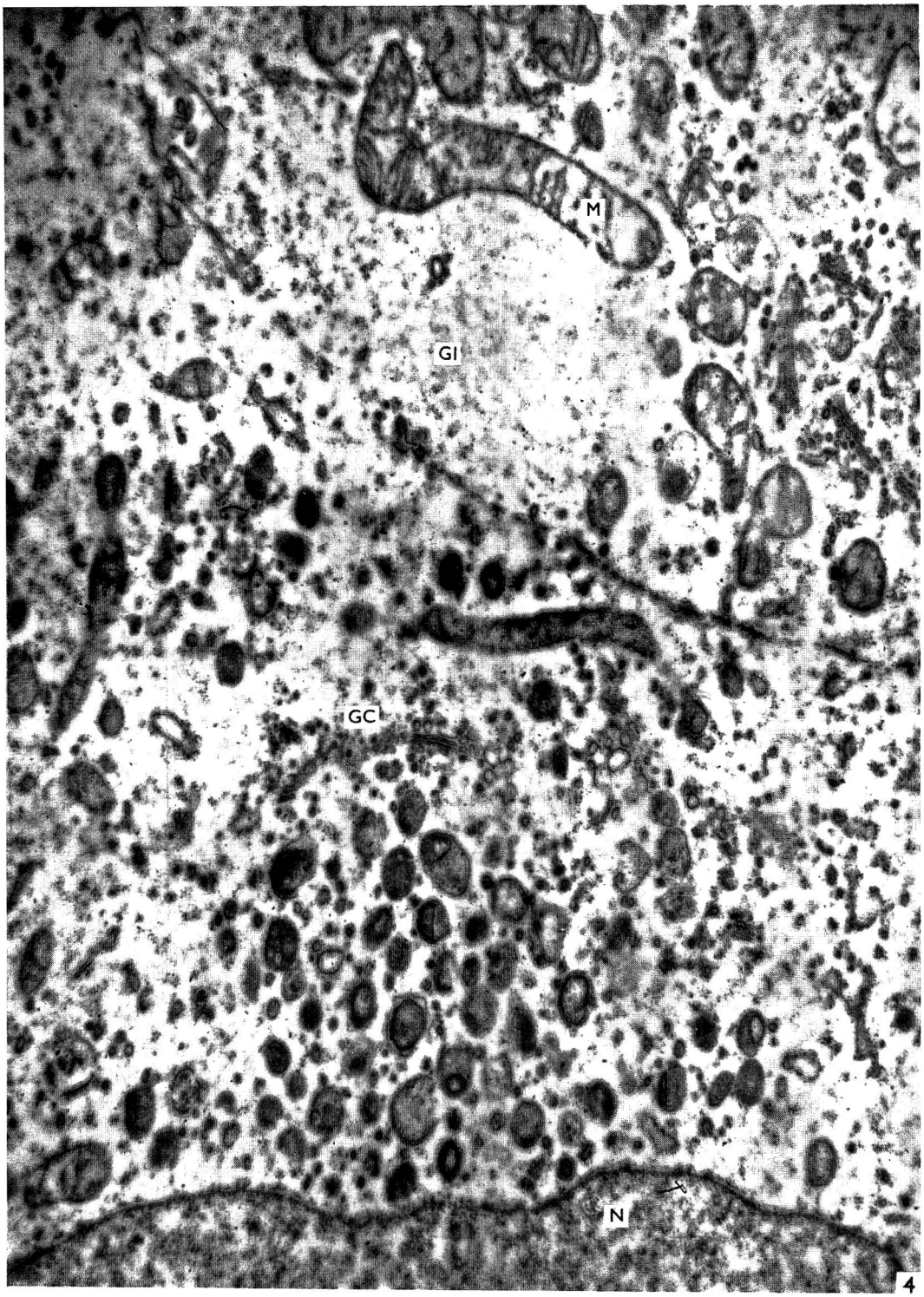

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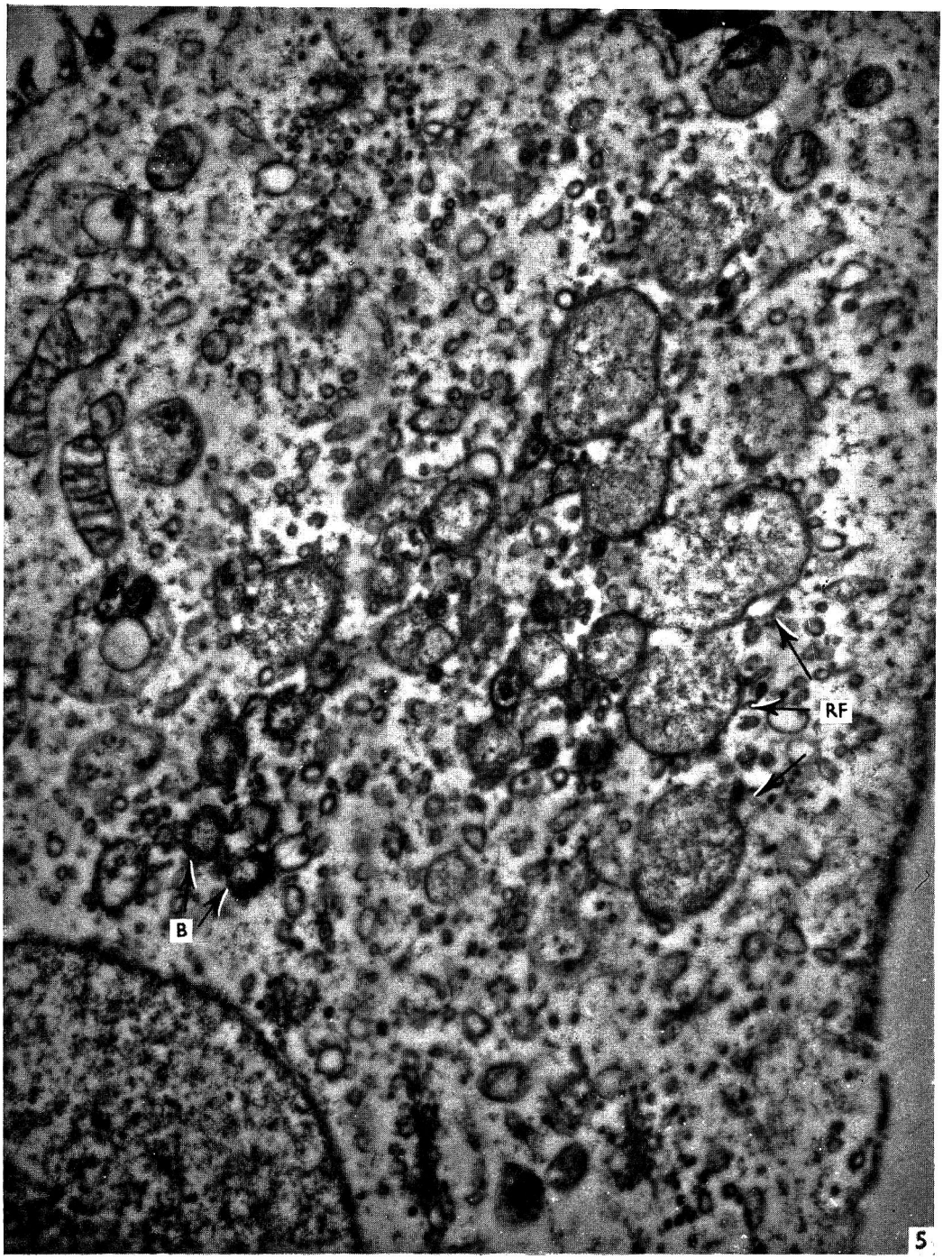

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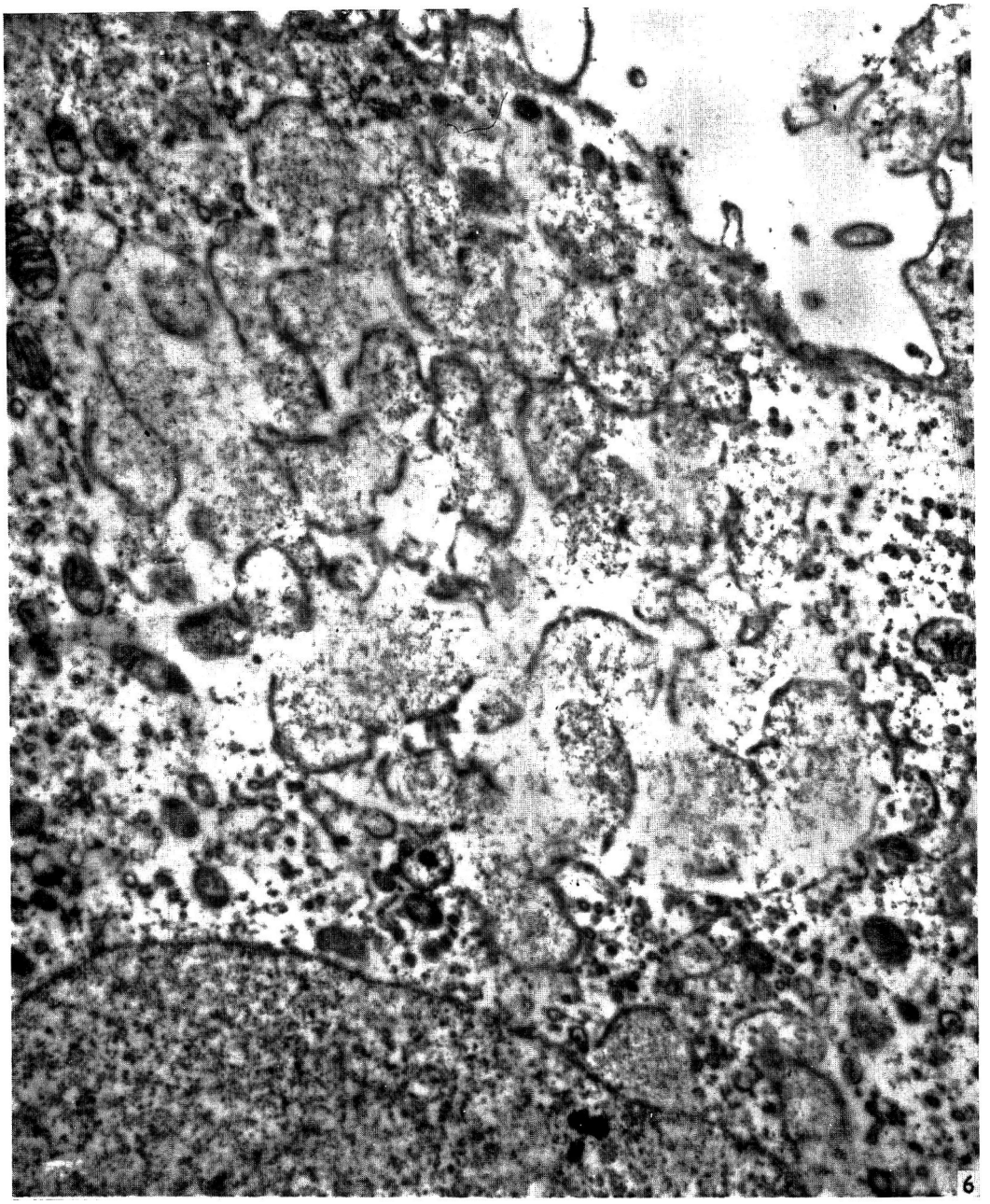

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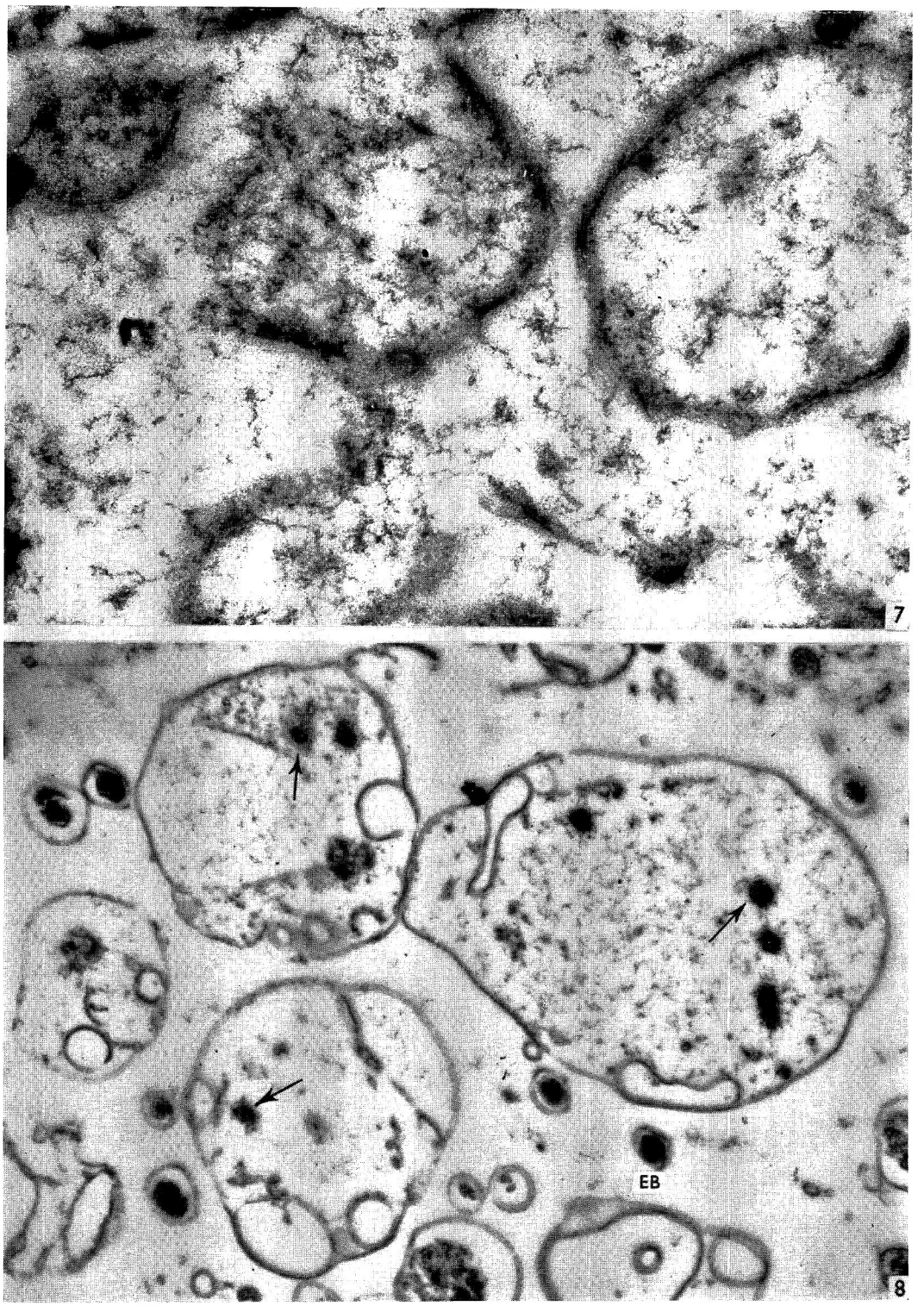

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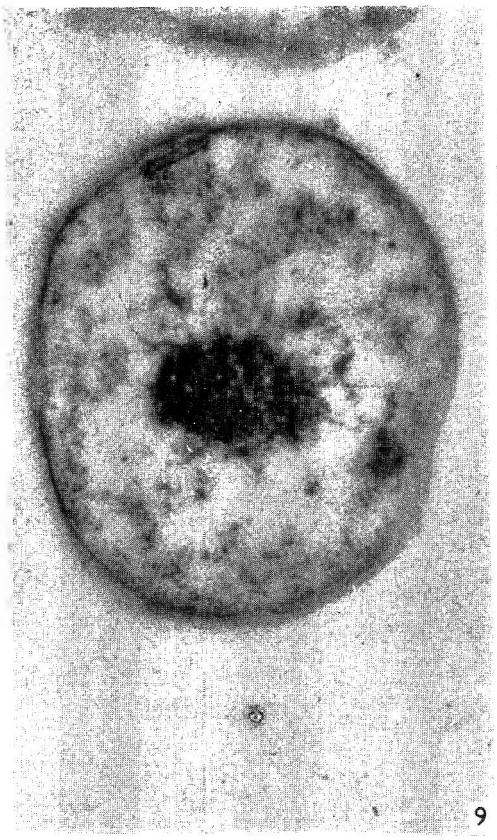
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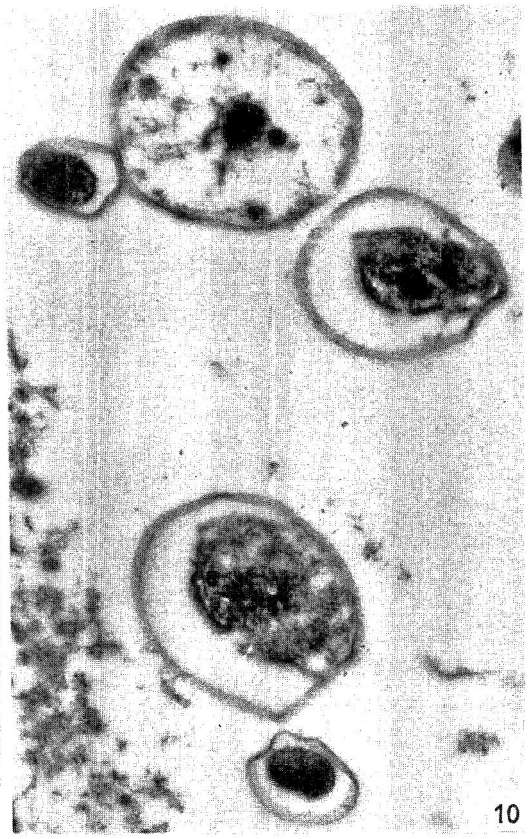
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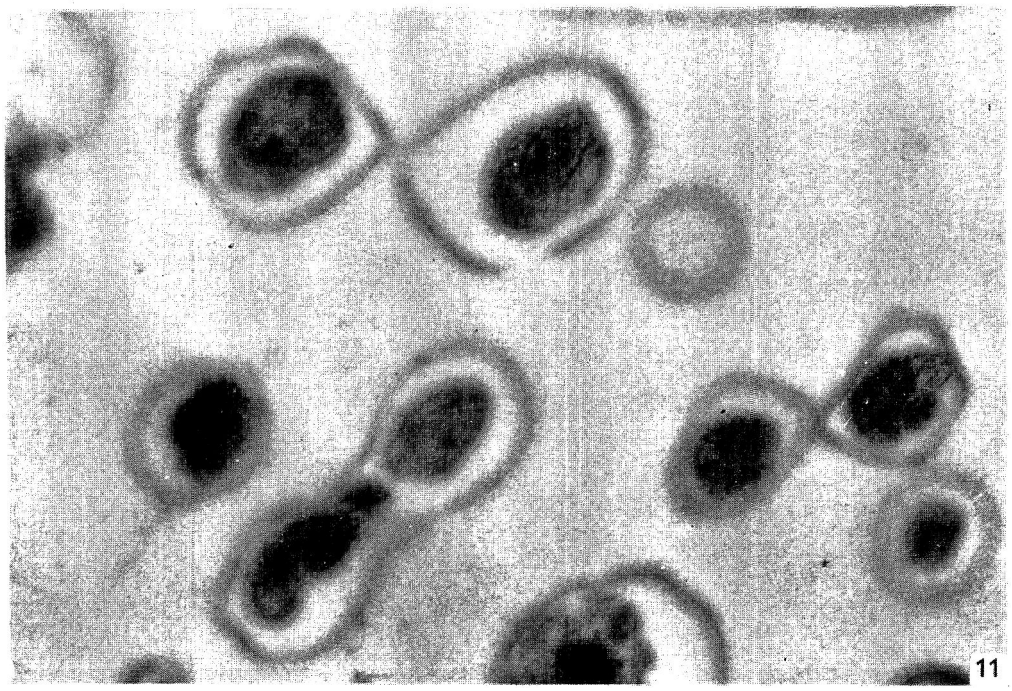
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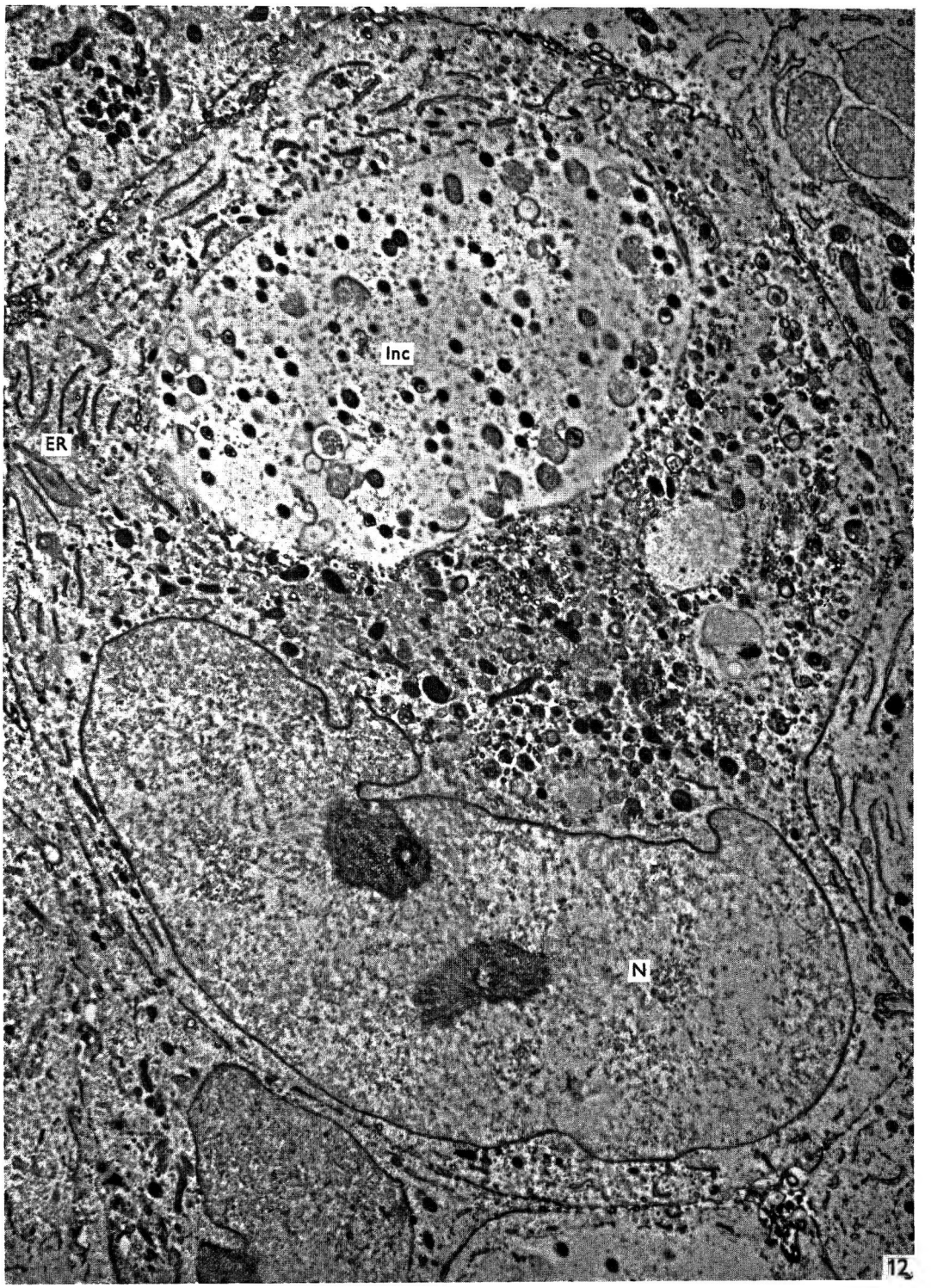

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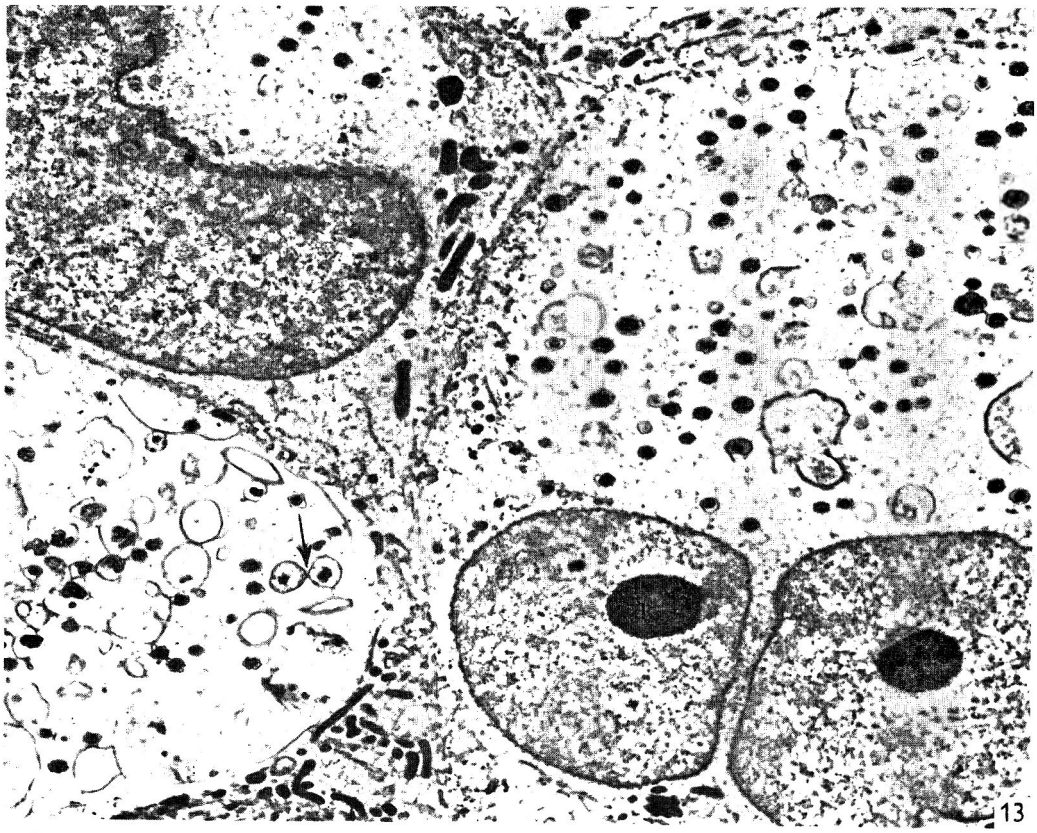

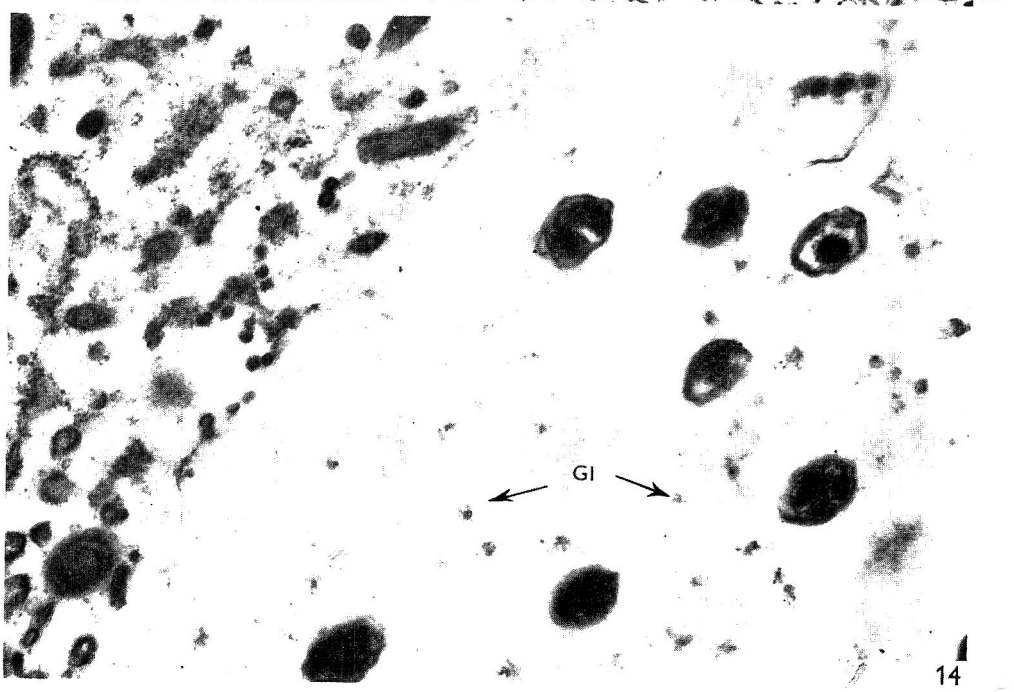

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PLATE 5

Fig. 7. A small part of a 24 hr. inclusion at higher magnification: the main visible component is a network of fine, electron dense fibrils. Remnants of the bilaminar membranes previously enclosing individual reticular foci are also present (× 70,000).

Fig. 8. Large developmental forms with complete limiting membranes, contained in a typical inclusion 48 hr. after inoculation. Note the presence of several fibrillar condensations (arrows) inside the developmental forms; in between the latter are a few apparently complete elementary bodies (EB) (× 20,000).

PLATE 6

Fig. 9. The structure of a discrete developmental form approximately 1 μ in diameter, at high magnification. Note the central fibrillar condensation, separated from the limiting membrane by a wide zone containing scattered granular elements (× 70,000).

Fig. 10. Profiles indicating the occurrence of unequal division, or budding, in a 48 hr. inclusion (× 35,000).

Fig. 11. Three paired profiles of relatively small developmental forms found in a section through a 72 hr. inclusion. They seem to indicate stages of division into equal halves (× 50,000).

PLATE 7

Fig. 12. Section through a cell containing an inclusion, 72 hr. after inoculation. The relationship of the inclusion (Inc) to the host cell is clearly displayed. Its contents include numerous elementary bodies; it is embedded in cytoplasm containing elements such as mitochondria and endoplasmic reticulum (ER). The cell nucleus (N) has been displaced but is structurally unaltered (× 5500).

PLATE 8

Fig. 13. Typical cells in a 72 hr. infected culture. In each, the cytoplasmic area is largely taken up by an enormous inclusion. The inclusion to the right in the micrograph has many mature elementary bodies; whereas that at the lower left still contains developmental forms of various sizes some of which show signs of division (arrow) (× 6000).

Fig. 14. Section showing the edge of a fully developed inclusion. No distinctive membrane is visible between the inclusion and the surrounding cytoplasm. Note that between elementary bodies the matrix contains moderately dense granular material (Gl), probably particulate glycogen (× 35,000).

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Mycological Examination of Dust from Mouldy Hay Associated with Farmer's Lung Disease

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SUMMARY

Small samples of hay were shaken in a perforated drum in a wind of 4·2 m./sec.; the liberated dust cloud was sampled with the cascade impactor for microscopical examination, and with the Andersen sampler for identification of organisms in culture. The results of testing batches of hay showed large differences in microbial content. Twenty-eight batches classed as 'good hay' gave up to 3 million spores/g. dry wt. hay, mainly *Aspergillus glaucus* with *Cladosporium* spp. and *Hemispora stellata*. Dust blown from seventeen batches of 'mouldy hay' differed greatly in composition and had from 5 to 250 million spores/g.: abundant forms included *A. glaucus*, *A. fumigatus*, *A. nidulans*, *Penicillium* spp., *Absidia* spp., *Mucor pusillus*, bacteria, many actinomycetes. Fourteen batches of mouldy hay associated with cases of 'farmer's lung' disease were generally similar in mould content to the batches of mouldy hay, but had many more spores of *Humicola* (*Monotospora*) *lanuginosa* and *A. fumigatus*, and were specially characterized by very many actinomycetes. Farmer's lung hays tended to neutrality (averaging pH 7·0, as compared with pH 5 to 6 of other hays), and were rich in thermophilic organisms, commonly with hundreds of millions of actinomycete spores/g., a large proportion of which grew readily at 60°. Hays associated with farmer's lung appear to have heated spontaneously to a higher temperature during maturation than the other hays. Dust from all hays contained particles of higher plants, but there were not obviously more in farmer's lung than in other hays. All types of spores were shown to be potentially able to penetrate to the deeper parts of the lung (especially the spores of actinomycetes, *A. fumigatus* and *H. stellata*), but whether these organisms play any part in the aetiology of the disease is unknown.

INTRODUCTION

Farmer's lung is a disease attributed to inhalation of dust from mouldy hay. This disease (reviewed by Frank, 1958) has been known since 1932 (Fuller, 1958), but interest in it has increased recently, perhaps because of changes in haymaking practice caused by the pick-up baler. The aetiology of farmer's lung is still obscure and the purpose of our work was to assess the possible role of the microbial components of the inhaled dust from mouldy hay. Previous research on spoilage of stored hay has been concerned mainly with self-heating (Browne, 1929) and spontaneous combustion (Miehe, 1930). The development of the microbial flora on grass composts has been studied by Webley (1947), Forsyth & Webley (1948) and by Eastwood (1948). Our work involved developing and testing of methods for sampling dust from hay, and examining farm hays (including both good hays and

hays associated with human and animal disease). The microfloral succession in experimental batches of hay baled at different moisture contents will be described later.

METHODS

Farmer's lung disease is attributed to the inhalation of dust from hay by farm workers when feeding mouldy hay to cattle in cowsheds. Hays were therefore tested by shaking in air and taking samples of the air for examination. The standard method of shaking in water and plating out dilutions was considered inappropriate because it would remove many kinds of particles which remain firmly attached to dry hay and which do not normally enter the air-borne dust fraction. (The washing method was, however, used for comparison with the blowing method in early tests.)

The method adopted after preliminary trials (Gregory & Lacey, 1963) uses reproducible conditions to give a standard test by which various batches of hay can be compared. Because the primary pathological changes occur in the deeper parts of the lung, it was considered useful to classify the dust into two fractions: (1) 'non-penetrating' larger particles which would be deposited mainly in the upper respiratory tract, nose, throat, bronchi and larger bronchioles; (2) 'penetrating' smaller particles which would be expected to reach the alveoli. Particles aerodynamically similar to unit-density spheres 5 μ diam. are considered to have a 50 % chance of penetrating to the alveoli, and this particle diameter was accordingly chosen to separate the two fractions, although the limiting size depends to some extent on the activity of respiration (Davies, 1952).

Hay sampling. Hay for test was normally received from the farm as whole or part bales and stored under cover at outdoor air temperatures (slices from bales were stored in large unsealed polythene bags). Samples for test were removed from the stored hay by pulling a handful from near the centre of the bale and carried to the laboratory in a small polythene bag with as little shaking as possible. (A 5-g. subsample was cut into 3 cm. lengths and occasionally stirred while in 250 ml. water for 15 min. before the pH value was measured electrometrically.)

Shaking in air. A sample of about 20–30 g. hay was tipped from the polythene bag into a cylinder of perforated zinc (2 mm. diam. perforations) with Perspex ends, which was mounted horizontally to rotate on bearings across a wind-tunnel of square cross-section with 29 cm. sides (Fig. 1). The cylinder was rotated during test at about 60 rev./min. by an electric motor to give a gentle 'tedding' action on the hay sample. Air was drawn down the wind-tunnel by a fan, a speed of about 4·2 m./sec. being adopted in routine tests. The dust cloud blown out of the cylinder travelled with the wind and reached the sampling position after a diffusion path of 1·2 m.

Air-sampling methods. A general study of hay dust must be based primarily on microscopic examination to reveal all its components, and must be supplemented by cultural examination for more precise identification of any organisms that can be grown in culture. For microscopic examination, air was sampled with a Casella model of the cascade impactor (May, 1945), connected to a vacuum pump drawing 20 l./min. through the orifice. Sampling at this rate was approximately isokinetic at the wind speed used. The cascade impactor accelerates the air-stream through four successively narrowing jets, impacting the entrained dust particles, graded into

four size fractions, on glass microscope slides made sticky with glycerol jelly applied on strips of transparent cellulose film. After exposure the strips were removed from the slides and the four dust traces mounted on a single microscope slide in glycerol jelly before examination (Pl. 1). In routine tests with the cascade impactor the sticky slides often had to be changed after the first minute of shaking, to avoid overloading the slides. The band of dust, which consisted mainly of fungal and actinomycete spores, was scanned and counted under a 3·75 mm. fluorite oil-immersion lens, the commoner types of particles being recorded on electrically operated counters. Recognition of the various types was facilitated by comparison with cultures and direct mounts from hay.

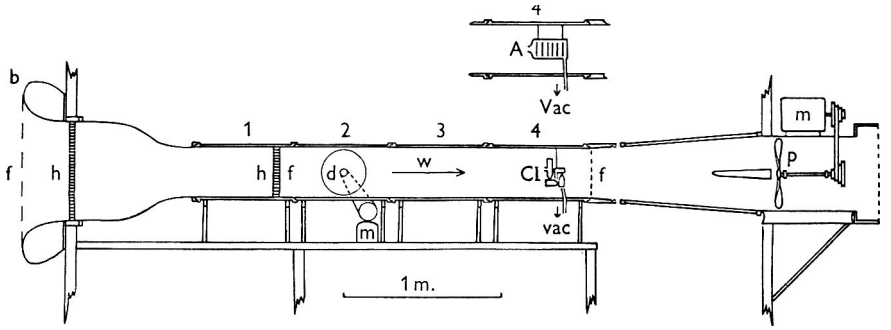

Fig. 1. Diagram of wind-tunnel showing position of sampling apparatus. 1–4, Perspex working sections; *b*, bell-shaped intake; h, paper honeycomb; f, filter screens; m, electric motors; p, fan; d, perforated zinc cylinder; CI, cascade impactor; A, Andersen sampler; vac, to vacuum pump; w, wind direction.

Calculation of numbers of spores blown from hay. Estimates of numbers of spores are given per g. dry wt. hay. Numbers of particles liberated per minute from a sample of hay were estimated as follows. The number of spores impacted from 20 l. air was first estimated by scanning the four dust traces from the cascade impactor. As 21·5 m.^{3} air/min. flowed down the wind-tunnel, the number caught in the impactor was multiplied by 1075 (= 21·5 m.^{3}/20 l.) and divided by the dry weight of the sample to give the total count/g. hay.

In the conditions used about 50 % of unit density spheres of 4·5 μ diam. would penetrate the first two jets of the cascade impactor, and 30 % of particles 0·5 μ diam. would be captured at the fourth stage. Accordingly particles captured behind jets 1 and 2 are classified as 'non-penetrating', and those captured behind jets 3 and 4 as 'penetrating', with respect to entry of the lung.

Preliminary tests of method. From results of preliminary tests with mouldy hay shaken in the wind-tunnel, a period of 3 min. blowing at a wind speed of 4·2 m./sec. was chosen as the standard for routine testing (Gregory & Lacey, 1963). Because conditions are not uniform within a bale of hay, the results from repeat tests differed. Visual counts of spores blown from successive samples from single bales of a good hay (L) and of a hay associated with a case of farmer's lung (H 44) illustrate this variability (Table 1).

Cultural examination. In preliminary tests several methods of sampling for the culturable fraction were tested, including the use of alginate wool filters and the liquid impinger. Both these methods ultimately placed the organisms in suspension

in water from which serial dilutions can be plated. However, with the mixture of organisms present in hay, the dilution stages proved completely unreliable; in one test a tenfold dilution series decreased numbers by only one fourth at each stage (compare Brierley, Jewson & Brierley, 1927). Dilution methods were therefore given up in favour of the Andersen sampler (Andersen, 1958). In this instrument the air enters a circular orifice and is drawn in succession through a series of six circular plates, each perforated with 400 holes through which spores are impacted directly on the surface of sterile medium in Petri dishes. Succeeding stages in the series have

Table 1. *Numbers of spores blown from successive samples taken from single bales of hay*

Good hay (L)			Farmer's lung hay (H 44)		
	Moulds	Actinomycetes (and bacteria)		Moulds	Actinomycetes (and bacteria)
Date	Spores (millions/g. dry wt. hay)		Date	Spores (millions/g. dry wt. hay)	
17. vi. 59	0·23	1·69	8. i. 60	67·9	400
19. vi. 59	0·07	0·58	11. i. 60	51·7	686
30. vi. 59	0·27	0·07	20. i. 60	34·1	205
7. vii. 59	0·19	0·24	30. iii. 60	153·0	969
6. x. 59	0·03	0·24	30. vi. 60	69·2	276

progressively smaller holes; the largest particles are deposited in the first dish and the smallest in the last. The Andersen sampler was placed horizontally on the axis of the wind-tunnel with the orifice facing the wind (Fig. 1), and was operated at 25 l. air/min. According to Andersen, the first two stages with larger holes retain the 'non-penetrating' fraction, while stages 3 to 6 retain the 'penetrating' fraction, as defined above. Flow into the sampler is non-isokinetic and, at the wind speed of 4·2 m./sec., the speed of air-flow through the orifice is only 16 % of the ambient tunnel wind speed; the largest particles in the non-penetrating range therefore are over-collected. On the other hand some of the large particles are lost by impaction on the front of the first perforated plate.

In an actual test of a sample of hay the cascade impactor was used during the first 3 min. of shaking, and then several sets of Petri dishes were exposed in the Andersen sampler during the fourth minute. After exposure, the sets of malt extract agar (2·0 % malt extract, 2·0 % agar, about pH 5·7), with 20 units penicillin/ml. and 40 units streptomycin/ml. to suppress bacteria, were incubated at 25° and 40°. Plates of half-strength Oxoid nutrient agar (pH 7·4) with 0·5 mg. actidione/ml. to suppress moulds were incubated at 25°, 40° and 60°. All plates were poured the day previously to allow the surface to dry. Each set of six plates was exposed in the Andersen sampler for 15 sec., giving 5 l. of air through the apparatus (occasionally to avoid overcrowding only 5 sec. were given). Although exposures were made during the fourth minute of shaking after the cascade impactor runs, when most of the spores were blown off, the plates were frequently overloaded. This was shown by the presence of the maximum possible number of 400 colonies/plate, many of them multiple infections for which no allowance has been made in Tables 3 and 5 to 7; counts from the Andersen sampler are therefore underestimates.

RESULTS

Examination of farm hays

Results of examining different batches of hay are presented in four groups: *a* good hays from a hay-drying experiment; *b* other good hays; *c* mouldy hays and hays associated with animal diseases; *d* farmer's lung hays. Classification into groups *b* and *c* was made on the results of the examination, but classification into group *d* was based on the diagnosis of farmer's lung in the patient by the physician. Table 2 shows the presence and abundance (over 2 million/g. hay) of spores in the different groups of hay as seen on the cascade impactor slides. Table 3 shows the presence and abundance (over twenty-five colonies isolated) of organisms isolated from the four groups of hay with the Andersen sampler.

Table 2. *Presence of spores counted on the cascade impactor slides*

(Brackets indicate number of samples containing over 2 million spores/g. dry wt. hay.)

	Experimental hays, group *a*	Good hays, group *b*	Mouldy hays, group *c*	Farmer's lung hays, group *d*
	No. batches tested			
	10	18	17	14
Actinomycetes	10 (3)	18 (5)	17 (15)	14 (14)
Acremoniella atra	0	0	1	2
Alternaria spp.	1	1	1	0
Aspergillus and *Penicillium* spp.	10 (7)	16 (5)	17 (7)	14 (7)
Chaetomium spp.	0	0	0	5
Cladosporium spp.	5	2	10	7
Epicoccum spp.	1	0	4	1
Humicola lanuginosa	0	0	9	13 (3)
H. stellata	0	0	2	3
Mucoraceae	2	7 (1)	13 (3)	11 (3)
Trichothecium roseum	0	2	3	5
Number of categories	6 (2)	6 (3)	10 (3)	10 (4)

Group a*: good hays from a hay-drying experiment* (*Table* 4). In July 1958 batches of hay were dried experimentally at the National Institute of Agricultural Engineering, Silsoe, Bedfordshire. Samples were obtained and tested in February and March 1959. Spores of *Aspergillus glaucus* and Penicillium type were the ones present in large quantities, and occurred in the swath dried hays (H7, 10, 11). A few spores of Cladosporium, Alternaria, Epicoccum and of Mucoraceae were also present. These hays never had more than 3 million actinomycete spores/g. hay. These batches of good hay were studied with alginate wool filters before the Andersen sampler was used. The fungi isolated were mainly mesophilic, the most numerous were *A. glaucus* (especially in H 10) and *Penicillium* spp. *Absidia* sp. was isolated from H 6 and 7, *A. fumigatus* from only H 8 and 13, and *Hemispora stellata* from H 5, 10, 11, and 13. Neither *Mucor pusillus* nor *Humicola lanuginosa* were isolated.

Group b*: other good hays* (*Table* 5). Samples classed as 'good hay' had very different microbial contents, ranging from H 33, of which the farmer who sent it was justly proud, to others yielding several million spores/g. hay. The pH values varied from 4·5 to 6·5 (av. of 13 hays, pH 5·9). The range of microbial content was

generally similar to the experimental hays of Table 4. Some samples, however, contained more actinomycete spores than moulds. *Aspergillus glaucus* was the most plentiful mould spore, especially in H 56, 58, 68 and 69. *Hemispora stellata* was

Table 3. *Isolation of organisms in culture*

(Brackets indicate number of samples with over 25 isolations.)

	Experimental hays, group *a*	Good hays, group *b*	Mouldy hays, group *c*	Farmer's lung hays, group *d*
	No. batches tested			
	10	18	17	14
Fungi				
Absidia (mainly *ramosa*)	2	15	13 (4)	13 (2)
A. corymbifera	0	2	0	0
Acremoniella atra	1	0	0	0
Alternaria spp.	1	0	3	0
Aspergillus spp. (total)	8 (1)	17 (6)	17 (10)	14 (10)
A. fumigatus	2	11	15 (5)	13 (5)
A. glaucus	7 (1)	15 (6)	12 (7)	12 (7)
A. nidulans	1	13	11 (4)	8 (1)
A. niger	0	0	5	0
A. ochraceus	0	0	1	0
A. terreus	0	2	6	4 (1)
A. versicolor	3	1	7 (2)	7 (1)
Botrytis spp.	5	1	3	3
Candida spp.	0	8	3	3
Cephalosporium sp.	1	0	1	0
Chaetomium sp.	1	3	5	4
Cladosporium sp.	2	15 (2)	13 (4)	13 (1)
Epicoccum sp.	2	1	1	0
Fusarium sp.	0	0	1	0
Geomyces type	3	2	0	1
Hemispora stellata	4	3 (3)	3 (1)	0
Humicola lanuginosa	0	6	4	9 (3)
H. stellata	0	0	0	1
Mucor pusillus	0	9	12 (3)	10 (1)
M. spinus	0	0	0	1
Paecilomyces sp.	0	6	3 (2)	9
Papularia sp.	0	1	1	0
Penicillium spp.	8	16	13 (4)	10 (1)
Pullularia sp.	0	1	1	1
Rhizopus sp.	0	1	1	1
Scopulariopsis brevicaulis	3	1	3	4 (2)
Sporendonema type	0	0	0	1
Sporotrichum sp.	1	0	0	3
Stachybotrys sp.	0	0	0	1
Stysanus spp.	1	0	1	0
Trichoderma viride	0	0	0	2
Trichothecium roseum	0	1	3 (1)	0
Yeasts	1	7	5	3 (1)
Sterile mycelium or unidentified	—	10	7	5
Number of categories	19 (1)	25 (3)	29 (11)	26 (12)
Bacteria				
24° orange	—	3	6 (4)	10 (3)
others	1	16 (5)	16 (3)	13 (4)
40° *Bacillus cereus-mycoides*	—	11	16 (1)	13 (3)
others	—	13	17 (3)	11 (3)
Number of categories	1	4 (1)	4 (4)	4 (4)

Table 3 (*cont.*)

	Experimental hays, group *a*	Good hays, group *b*	Mouldy hays, group *c*	Farmer's lung hays, group *d*
Actinomycetes				
24°	2	15	17 (8)	13 (8)
40° whitish	—	15 (12)	17 (10)	15 (9)
blue	—	2	8 (2)	11 (4)
grey	—	10 (1)	12	12 (3)
thin	—	3	3 (1)	0
small	—	4 (2)	11 (6)	13 (12)
60° white	—	11 (3)	13 (7)	13 (11)
small	—	0	4 (3)	9 (9)
Number of categories	1	7 (4)	8 (7)	7 (7)
Total number of categories	21 (1)	36 (8)	41 (22)	37 (23)

Table 4. *Microbial content of good hay* (*group* a) *artificially dried at N.I.A.E., Silsoe, Bedfordshire, in July* 1958

		Visual examination	
		Moulds	Actino-mycetes
		Spores (millions/g. dry wt. hay)	
Hay ref. no.	Treatment		
H 5 (D. 1)	Tunnel dried	4·5	1·1
H 6 (D. 2)	Hut rack dried	2·9	0·8
H 7 (D. 3)	Swath dried	32·3	2·4
H 8 (C. 1)	Tunnel dried	0·1	0·6
H 9 (C. 2)	Hut rack dried	2·1	0·9
H 10 (C. 3)	Swath dried	9·2	1·3
H 11 (C. 4)	Swath dried	10·3	2·5
H 12 (E. 1)	Barn dried	5·6	1·6
H 13 (E. 2)	Hut rack dried	2·8	0·5
H 14 (E. 3)	Swath dried	6·9	3·0

seen in H 37 and 69, and in H 56 0·68 million spores/g. hay were counted. Mucoraceae spores were seen in only seven of these hays (Table 2) and only in H 17 reached 2 million/g. The hays from groups *a* and *b* contain fungi, such as Cladosporium, Epicoccum, Helminthosporium and Alternaria, which are the normal constituents of fine weather air-spora; but hays of poorer quality had moulded with *A. glaucus*, *H. stellata* and *Penicillium* spp. In culture, the only abundant moulds were *A. glaucus* (H 37, 56, 58, 68, G, Y), *Cladosporium* sp. (H 67, 68), and *H. stellata* (H 37, 56 and 69 with 14,000 viable spores/g.). A few *A. fumigatus* were isolated from all except H 33, 37, B, G, and N, of *Absidia* spp. from all except H 19, 33, and 71, and of *Mucor pusillus* from half. Very few *Humicola lanuginosa* were isolated from H 69, 71, B, G, L and Y. Bacteria and actinomycetes were isolated from most hays but even when shown 'abundant' (Table 6) were many fewer than in hays of groups *c* and *d*.

Group c*: mouldy hays* (*Table* 6). These included batches associated with diseases of farm animals and various other samples sent to us as mouldy, but excluding any

samples sent as implicated in producing farmer's lung disease; the latter are dealt with in group *d*. The pH value ranged from 4·0 to 8·3; the average of 13 hays (pH 6·5) was very similar to that of the good hays of group *b*. The hays of group *c* had very different microbial contents, and spores of moulds or actinomycetes (or both) usually numbered between 10 and 100 million/g. Aspergillus and Penicillium type spores were numerous in H 27, 49, 59, 63, 66, 74, and 79, Mucoraceae were abundant in only H 27 and 76. *Humicola lanuginosa* was present in H 1, 27, 40, 41, 49, 59, 61, 63 and 66, *H. stellata* in H 27 and 41.

Table 5. *Microbial content of good hay samples, group* b

Hay ref. no.	Origin	pH value	Visual examination (Moulds Spores (millions/g. dry wt. hay))		From culture (Moulds Spores (thousands/g. dry wt. hay))		
			Moulds	Actinomycetes	Moulds	Actinomycetes	Bacteria
H 17	Weybridge, Surrey, as fed to cows at Central Veterinary Laboratory	—	2·75	1·34	—	—	—
H 19	Plumpton, Sussex (normal hay fed to cows on a farm where mycotic abortion had occurred)	—	0·77	1·68	—	—	—
H 33	Leckhampstead, Newbury, Berks.	—	0·17	0·22	—	—	—
H 37	Dulverton, Som. (1959 hay 'inactive'; cf. H 36 (Table 7))	6·4	2·60	2·90	26·6	4·30	0·41
H 38	Weymouth, Dorset (associated with case of sarcoid)	6·5	3·2	2·4	—	—	—
H 56	Lympsham, nr. Weston-super-Mare, Somerset (associated with non-seasonal allergic hay fever and asthma)	6·2	3·5	1·0	17·6	5·86	3·08
H 58	Banwell, Somerset	6·3	5·44	8·17	7·39	7·87	0·76
H 67	Beacon, Honiton, Devon, old ley, 1961	6·3	0·21	0·67	1·73	0·19	1·12
H 68	Luppitt, Honiton, Devon, 2nd year ley, 1961	6·2	6·89	0·25	17·9	0·30	0·28
H 69	Luppitt, Honiton, Devon, 3rd year ley, 1961	5·4	6·23	0·19	9·36	0·75	0·66
H 71	Knighton, Radnorshire, 2nd year ley, 1961	6·2	0·23	1·89	1·18	0·72	0·85
H 72	Knighton, Radnorshire, meadow hay, 1961	6·2	0·81	0·72	1·99	1·06	2·84
H 73	Knighton, Radnorshire, 1st year ley, 1961	4·5	0·11	0·41	0·97	0·36	1·25
B	Rothamsted, Harpenden, Herts., 1961	6·0	0	0·08	0·30	0·37	1·58
G	Rothamsted, Harpenden, Herts., 1961	6·3	0·76	0·07	13·9	0·94	0·82
L	Rothamsted, Harpenden, Herts., 1959	—	0·41	0·54	—	—	—
N	Rothamsted, Harpenden, Herts., 1959	—	2·21	5·6	—	—	—
Y	Rothamsted, Harpenden, Herts., 1960	5·2	1·43	2·16	4·40	4·78	0·73

In culture, many thermophilic moulds were abundant: *Absidia* spp. (H 61, 75, 76, 79), *Aspergillus fumigatus* (H 1, 63, 66, 76, 79), *A. nidulans* (H 1, 66, 76, 79), *Mucor pusillus* (H 59, 66, 79) and *Paecilomyces* sp. (H 59, 75). Abundant mesophilic moulds were: *A. glaucus* (H 1, 46, 49, 53, 61, 66, 74), *A. versicolor* (H 59, 74), *Cladosporium* sp. (H 49, 53, 59, 74), *Hemispora stellata* (H 74), *Penicillium* spp. (H 1, 74, 76, 79) and *Trichothecium roseum* (H 79). *Humicola lanuginosa* was isolated from H 1, 40, 53 and 63. Bacteria and actinomycetes were isolated from all samples. Bacteria isolated at 24° were abundant in H 1, 16, 59, 61, 66 and 74, and at 40° in H 1, 16, 59 and 66. Actinomycetes isolated at 24° were abundant in H 1, 16, 27, 59, 61, 63, 76 and 79; at 40° in H 1, 16, 27, 41, 59, 61, 63, 75, 76 and 79; and at 60° in H 1, 16, 59, 61, 63, 75 and 79.

Group d*: farmer's lung hays* (*Table* 7). Fourteen samples of hay associated with cases of farmer's lung were examined between 1958 and 1961. Some of these were browner than normal hay, but they differed from the typical brown hay described by Truninger (1929) which was usually acid (pH 4·4–5·1). Characteristically farmer's lung hay is less acid than the 'good' or 'mouldy' hays of groups *b* and *c*, between pH 6·0 and 7·5 (average of 12 hays, pH 7·1). The outstanding character of group *d* hay was the large content of actinomycete spores, over 60 million/g. (except H 45 with only 5 million). Fungus spores are often abundant. Aspergillus

Table 6. *Microbial content of mouldy hays and hays associated with animal diseases, group* c

Hay ref. no.	Origin	pH value	Visual examination (millions/g. dry wt. hay)		From culture (thousands/g. dry wt. hay)		
			Moulds	Actinomycetes Spores	Moulds	Actinomycetes Spores	Bacteria
	Relatively good hays associated with animal diseases						
H 46	Haslemere, Surrey (mycotic abortion in cows)	6·1	1·1	4·0	3·44	2·64	1·12
H 49	Norwich, Norfolk (mucormycotic abomasal ulcers in Jersey calves)	6·2	1·1	4·6	9·49	1·23	1·52
H 53	Reading, Berks. (buccal ulceration in cows)	6·0	1·0	4·0	21·4	3·92	0·5
H 75	Horsham, Sussex, 1960 hay (mycotic abortion in cows)	4·0	0·8	1·36	3·25	4·18	0·4
	Mouldy hays associated with animal diseases						
H 1	Reading, Berks. (acute pneumonia of calves)	—	71·5	80·2	10·83	56·3	0·92
H 16	Cambridge (aspergillosis of lambs)	—	0·76	190·0	4·32	79·8	25·2
H 74	Horsham, Sussex, 1961 hay (mycotic abortion in cows)	5·3	15·1	2·16	4·45	0·34	0·98
H 76	Horsham, Sussex, 1960 hay (mycotic abortion in cows)	5·3	42·0	8·47	48·2	58·3	0·63
H 79	Basingstoke, Hants. (coughing in Guernsey cows)	5·7	19·5	1·34	57·9	0·54	0·21
	Other mouldy hays						
H 27	Liskeard, Cornwall	—	100·0	49·6	—	—	—
H 40	Brampton, Cumb., 1958 ley hay	4·5	4·2	251·0	—	—	—
H 41	Brampton, Cumb., 1958 old land meadow hay	8·3	4·1	193·0	—	—	—
H 42	Brampton, Cumb., 1958 meadow hay	7·4	2·2	205·0	—	—	—
H 59	Banwell, Somerset, old pasture hay	6·8	7·4	41·9	19·5	31·3	22·7
H 61	Berkeley, Glos.	7·5	2·6	28·0	11·2	60·9	13·0
H 63	Aberystwyth (inactive with patient of H 64; Table 7)	6·1	4·5	25·6	4·55	39·8	1·03
H 66	Rothamsted, Harpenden, Herts., 1961 hay	—	27·8	3·95	4·56	0·24	23·2

and Penicillium spores were abundant in H 2, 30, 31, 32, 44, 54 and 55. *Humicola lanuginosa* was abundant in H 18, 43 and 44, and the Mucoraceae in H 30, 31 and 44. *H. stellata* was seen in H 31, 43 and 52, and *Trichothecium roseum* in H 2, 30, 31, 44 and 64.

In culture, many thermophilic moulds were abundant: *Absidia* spp. (H 30, 31), *Aspergillus fumigatus* (H 30, 44, 52, 54, 64), *A. nidulans* (H 30), *A. terreus* (H 30), *Humicola lanuginosa* (H 18, 43, 64) and *Mucor pusillus* (H 44). Mesophilic moulds

that were abundant were: *A. glaucus* (H 2, 31, 32, 36, 44, 45, 65), *A. versicolor* (H 2), *Cladosporium* sp. (H 30), *Penicillium* spp. (H 30), *Scopulariopsis brevicaulis* (H 2, 31) and a yeast (H 32). *Humicola stellata* was isolated from H 54 and a thermophilic *Sporotrichum* sp. from H 18, 44 and 52. Abundant bacteria were isolated at both 24° and 40° from H 30, 31, 32, 44 and 64. Actinomycetes isolated at 24° were abundant in H 2, 30, 31, 36, 44, 52, 54 and 64; at 40° and 60° in all hays except H 45 (and H 52 which was not tested at 60°). Although H 45 seems out of place in this group, the diagnosis of farmer's lung was well established and the discrepancy may

Table 7. *Microbial content of farmer's lung hays, group* d

Hay ref. no.	Origin	pH value	Visual examination (millions/g. dry wt. hay)			From culture (thousands/g. dry wt. hay)		
			Moulds	Actino-mycetes Spores	*H. lanuginosa*	Moulds	Actino-mycetes Spores	Bacteria
H 2	Witney, Oxfordshire (acute thresher's lung)	7·5	11·0	134·0	0·08	11·3	70·3	1·01
H 18	Near Aberystwyth	7·0	3·6	168·0	1·10	4·84	16·2	4·72
H 30	Whitchurch, Shropshire	6·0	86·2	132·0	13·8	143·2	103·3	53·85
H 31	Broadwoodkelly, nr. Winkleigh, Devon (1958 hay)	7·6	12·0	363·0	0·64	6·24	60·7	12·52
H 32	Broadwoodkelly, nr. Winkleigh, Devon (1959 hay)	7·3	7·8	480·0	0·97	18·7	85·7	9·45
H 36	Dulverton, Som. (1958 hay 'active') cf. H 37, Table 5	6·6	9·1	91·3	0	13·4	216·6	5·78
H 43	Awliscombe, nr. Exeter, Devon	6·6	7·4	1232	2·4	14·4	152·1	1·48
H 44	Inwardleigh, nr. Okehampton, Devon	7·4	102·0	827·0	11·2	101·8	208·3	21·01
H 45	Lampeter, mid-Cardiganshire (farmer's lung of young lad)	6·7	1·0	5·0	0·1	3·23	1·57	0·68
H 52	Mid-Wales	—	1·5	633·0	0·24	10·5	28·0	43·67
H 54	Longnor, Staffordshire	7·4	16·6	78·3	2·87	4·67	24·7	2·28
H 55	Cardiff	—	12·4	123·0	0	3·75	23·2	0·57
H 64	Talybont, nr. Aberystwyth, 1960 'active' hay (cf. H 63, Table 6) (farmer's lung for 20 years)	7·5	2·3	175·0	0·83	9·14	81·5	4·10
H 65	Matlock, nr. Chesterfield, Derbyshire	7·2	1·4	64·0	0·12	8·04	37·2	2·20

mean either that the sample sent from the farm was taken accidentally from the wrong batch of hay, or the sample sent may have been unrepresentative of the batch because of the variability within the batch from uneven drying.

Proportion of thermophilic organisms

In comparison with good hay, the mouldy hay group *c* and the farmer's lung group *d* were characterized by many more thermophilic moulds and actinomycetes, as shown by incubation of Andersen sampler plates at different temperatures. These average values (Fig. 2), however, were calculated from individual hays which differed among themselves over a wide range of values, were uncorrected for multiple infection, and included some organisms which grow at more than one temperature. The mucoraceous moulds were more numerous at 40° than at 24°, and in mouldy

hays (group *c*) than in farmer's lung hays (group *d*). *Aspergillus fumigatus* was isolated in larger numbers at 40° than at 24°, and *Humicola lanuginosa* only at 40°. The relatively few bacteria were isolated mainly at the lower temperatures. The most striking effect was the very large increase in thermophilic actinomycetes in farmer's lung hays (group *d*).

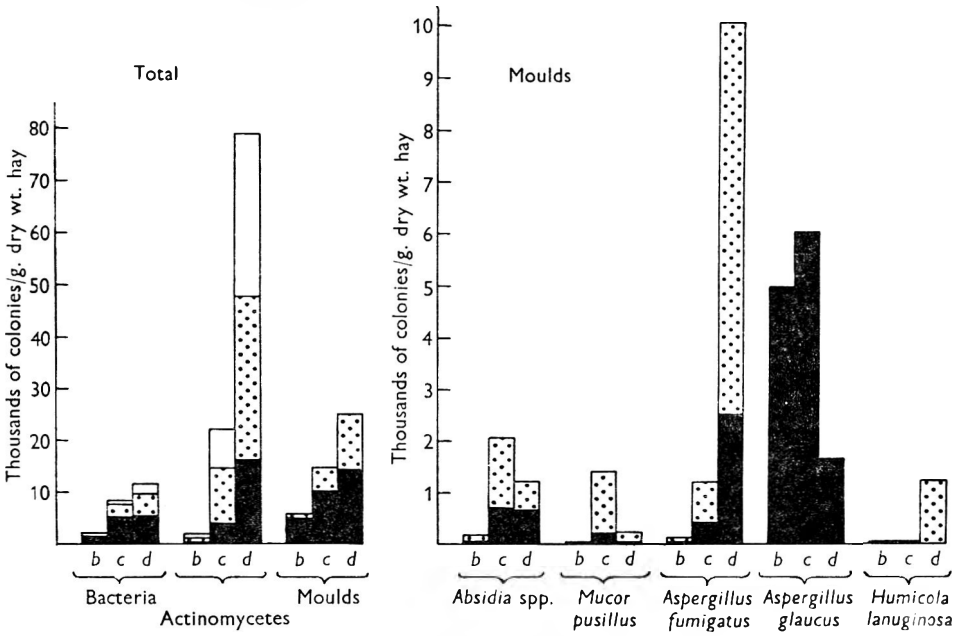

Fig. 2. Numbers of organisms isolated at 24° (solid), 40° (dotted) and 60° (open), averages for: (*b*) good hays; (*c*) mouldy hays; and (*d*) farmer's lung hays.

Other components of dusts

The visual inspection of the first slide from the cascade impactor showed a good deal of coarse material other than the spores of micro-organisms. This material included fragments of plants, plant hairs, cell walls, faecal pellets of mites (packed with mould spores), cast skins of mites, as well as whole conidial heads of Aspergillus and sporangia of Mucoraceae (the last help to substantiate identification of the Mucoraceae spore type among the finer fractions). Few of these particles penetrated beyond the first stage of the impactor and material deposited in the later stages was predominantly microbial spores. The better hays seemed to have more non-spore dust than the mouldy hays, possibly because the process of becoming mouldy tended to bind the loose particles in a weft of mycelium. Plate 1 shows typical fields of cascade impactor slides of a good hay (G) and a farmer's lung hay (H 44).

Possibility of penetration of particles into lungs

The visual and cultural methods of assessing potential alveolar penetration in general agreed. For the two methods, Fig. 3 shows graphically the % of particles retained by the four cascade impactor slides and the six Andersen sampler plates. The values obtained are combined averages of results from mouldy and farmer's lung hays (groups *c*, *d*). According to visual estimates, only 8 % of spores of *Humi-*

cola lanuginosa and the Mucoraceae penetrated beyond stage 2 of the cascade impactor, but 43 % of Aspergillus and 55 % of actinomycete spores were 'penetrating', by the criteria mentioned under Methods. According to cultural tests, however, a larger proportion of spores were 'penetrating', namely, 60 % of the Mucoraceae and over 80 % of the other groups. This discrepancy is partly attributed to the fact that the first two plates received clumps of spores (or even whole sporangia as in stage 1 of Mucoraceae) which, although they contained many spores, produced only one colony. Further, discrimination between single spores of Mucoraceae and members of the Aspergillus–Penicillium type was sometimes difficult; although spores of *Absidia* spp. are easily recognized, some of *Mucor pusillus* may have been misclassified, suggesting that the Mucoraceae types (Fig. 3) appeared to be less

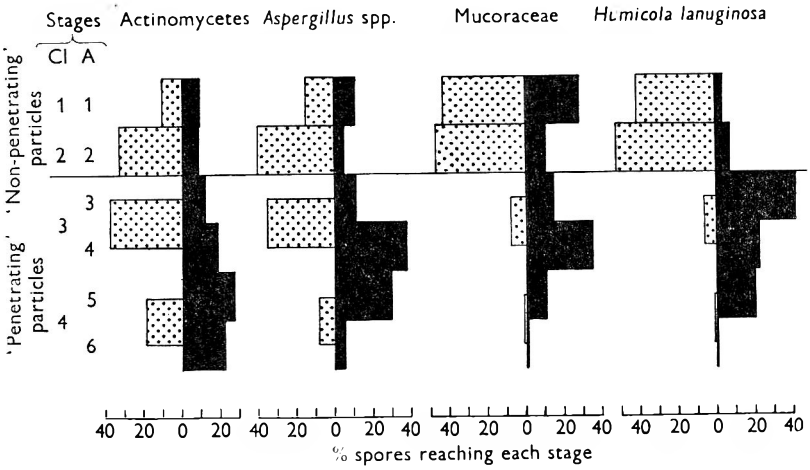

Fig. 3. Percentage penetration of spores to the four stages of the cascade impactor (CI; dotted), and to the six stages of the Andersen sampler (A; solid), average of groups *c* and *d*.

penetrating than they actually were. Spores of *Humicola lanuginosa* are about 9 μ diam. and the maximum proportion of 42 % were found on stage 3; those of Aspergillus are about 4 μ and the maximum of 38 % was found on stage 4, but 30 % penetrated as far as stage 5. *Hemispora stellata* has smaller spores, most were found on stage 5 and hardly any on stage 6. Spores of the actinomycetes penetrated deeply, 27 % reaching stage 5, and 23 % stage 6. Evidently many single spores of fungi and the majority of actinomycete spores could penetrate to the alveoli.

DISCUSSION

Our standard method of examining hays gives results that are reproducible enough to characterize batches of hay. Standardizing the humidity of the air in the wind tunnel would be a desirable improvement, as Zoberi (1961) found that humidity greatly affected the numbers of dry spores liberated into wind; but since a bale of hay is a concretion of micro-environments, errors from differences between bales and between parts of a single bale (Table 1) are likely to outweigh the humidity factor. Because the dust load is so easily shaken out, it was not considered feasible to work with a subsample taken after thoroughly mixing several separate samples.

Incidental to the main object of the work, the Andersen sampler proved an excellent tool for isolating thermophilic actinomycetes from natural substrata. Dilution in air, instead of in water by the traditional manner, and impacting the spores from suspension in air directly on to the previously dried surface of half-strength nutrient agar containing actidione, prevented contamination by bacteria and moulds, and on incubation overnight at 60° gave numerous colonies of thermophilic actinomycetes (Gregory & Lacey, 1962).

The proportion of spores seen on cascade impactor slides which was actually obtained in culture with the Andersen sampler was always small, and averaged about one in a thousand, even after allowing for the fact that two-thirds of the spore load had been lost from the sample during the 3 min. blowing period for the cascade impactor run before the 15 sec. run with the Andersen sampler started (Table 8). Reasons for this small % recovery in culture include the following: (1) no correction was applied for multiple infection, so that the counts tended to be gross underestimates, especially when nearly the maximum number of 400 colonies are recorded on one plate; (2) the media, aeration conditions and isolation temperatures may have been unsuitable for some organisms; (3) slow-growing organisms may have been overgrown or antagonized on the crowded plates; (4) many spores may have been dead. Table 8 shows that the percentage obtained in culture was larger with the better-quality hays. From these tests it is clear that both the mouldy and farmer's lung hays (groups *c* and *d*) are important natural reservoirs of several moulds reported to be pathogenic to man or animals, e.g. *Absidia ramosa* and *A. corymbifera*, *Aspergillus fumigatus*, *Hemispora stellata*, *Mucor pusillus*, *Scopulariopsis brevicaulis* and *Stachybotrys* sp.

Table 8. *Numbers obtained in culture on Andersen sampler plates as percentage* (%) *of spores recorded visually on cascade impactor slides*

Hays	Moulds	Actinomycetes and bacteria
	%	
Group *b*	0·34	0·22
Group *c*	0·10	0·11
Group *d*	0·14	0·03

The tests show it possible to characterize farmer's lung hays (group *d*). They had clearly heated spontaneously and become mouldy during maturation, and tended to be neutral in contrast to the low pH values of good hays or other mouldy hays examined. They had many more thermophilic organisms, including *Aspergillus fumigatus*, and *Humicola lanuginosa*, than other mouldy hays, and were rich in thermophilic actinomycetes (especially at 60°), which were often seen microscopically to be growing vigorously on the hay and on mould hyphae and spores. Several hays of the mouldy group *c* might well have been classified as farmer's lung hays had they come into contact with a susceptible patient. In spite of their abundance, and evidence of their potential ability to penetrate to the alveoli, it is uncertain whether any of these organisms play any direct part in the aetiology of farmer's lung disease, but the possibility that the actinomycetes are active merits serious attention in view of the occurrence of the disease after inhalation of white dust from mouldy hay reported by Campbell (1932) and Frank (1958, p. 202).

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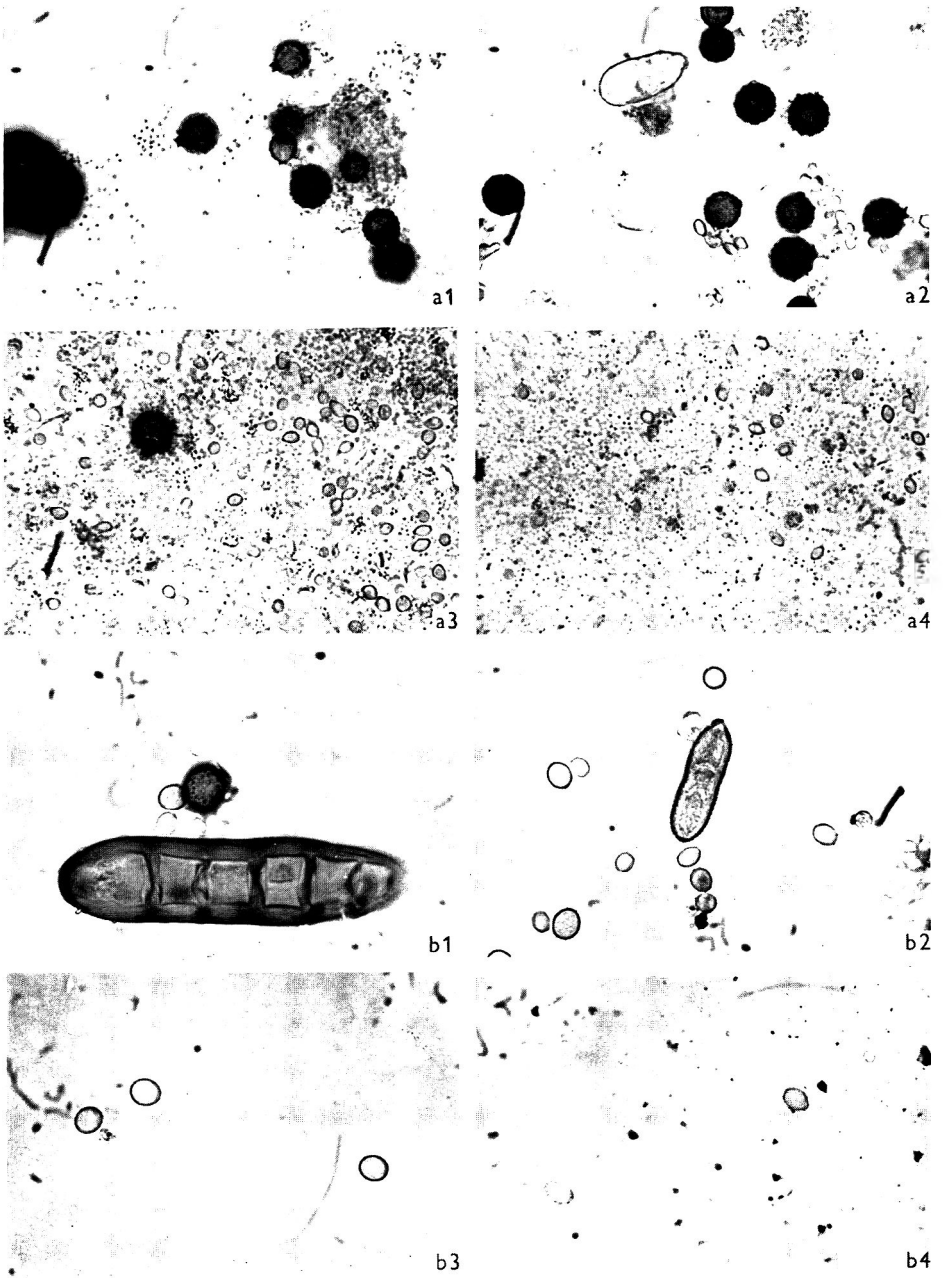
EXPLANATION OF PLATE 1

Photomicrographs (× 600) from cascade impactor slides 1, 2, 3 and 4 to compare a good hay with a farmer's lung hay.

a Farmer's lung hay from Devon (H 44).

b Good hay from Rothamsted Experimental Farm (G, 1961).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Recovery of Deoxyribonucleic Acid from the Effects of Alkylation

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SUMMARY

The transforming activity for an indole locus of DNA extracted from a methionine-requiring strain of *Bacillus subtilis* was decreased about tenfold per unit of DNA by treatment of organisms with 0·025 M-methylmethanesulphonate for 30 min. at 37°. The treatment decreased the viable count to one tenth of the original and induced a reversion frequency of 10^{-5}. Transforming activity of DNA prepared from alkylated organisms was sensitive, to heating at 50° as compared to DNA extracted from non-alkylated organisms. Methylation and heating of DNA *in vitro* did not inhibit its uptake by competent organisms. Incubation of treated and non-treated organisms in amino acid supplemented medium led to an increase in extractable transforming activity. A period of incubation which led to a 34% increase in DNA content more than doubled the transforming activity obtainable from methyl methanesulphonate treated organisms. The heat sensitivity of transforming DNA from alkylated organisms decreased after incubation of the organisms. Synthesis of DNA in the absence of a net DNA increase was demonstrated in methyl methanesulphonate treated organisms since thymidine-2-^{14}C was incorporated without lag from the start of incubation into an alkali-stable hot acid-soluble form. The experiments are interpreted to mean that alkylated DNA can serve as a template for the replication of DNA of normal activity.

INTRODUCTION

One of the questions to be answered about any mutagenic agent is whether its reaction with the genetic material (deoxyribonucleic acid, DNA) of an organism is direct or indirect. If the mutagen methylmethanesulphonate reacted directly with DNA for example, it would be possible to show that immediately after treatment of organisms with alkylating agent, the DNA of the organism became heat sensitive (Strauss, 1962*a*). Since such a determination would require a biological assay for DNA activity, the experiments would have to be done with an organism capable of undergoing transformation. It seemed likely that the system described by Spizizen (1958) and Anagnostopoulos & Spizizen (1961) with *Bacillus subtilis* would be particularly satisfactory because of the possibility of obtaining auxotrophic mutants of this organism similar to those of *Escherichia coli*. The experiments to be described here show that treatment of *B. subtilis* with an alkylating agent resulted in heat sensitization of the DNA extractable from the treated organism. In addition, the DNA of methylated organisms gradually lost its abnormal heat sensitivity as these organisms were incubated in growth medium. Alkylated DNA appears to serve as a template for the production of normal DNA.

METHODS

Several strains of *Bacillus subtilis* were used. The first, a methionine-requiring mutant, was obtained by replica plating from a culture of the prototrophic *B. subtilis* which had been treated with ethylmethanesulphonate. This strain was used as a DNA donor for the transformations described in this paper with the exception of transformation with ^{32}P labelled DNA when a wild type donor was used. The methionine-requiring strain was selected because it could be induced to revert by treatment with methylmethanesulphonate (Fig. 1) as well as with the ethyl derivative, and because it would serve as a recipient strain for transformation as well as being a satisfactory donor. The strain was heat sensitive after methylation

Table 1. *Loss of the differential heat sensitivity due to alkylation upon incubation of a methionine-requiring mutant of* Bacillus subtilis

A washed suspension of cells from an overnight culture was treated for 30 min. at 37° with 0·025 M-methylmethanesulphonate in salts solution (0·043 ml. methylmethanesulphonate/20 ml. of salts solution). After treatment the organisms were washed and suspended in 10 ml. of salts solution. Two ml. were then added to flasks containing 38 ml. of minimal medium (Spizizen, 1958) supplemented with 0·02 % casein hydrolysate, and 50 μg./ml. each of L-methionine and L-tryptophan. The flasks were incubated for the indicated time at 37° with shaking and then the contents were collected by centrifugation and washed with salts solution. The organisms were suspended in 5 ml. of salts solution and plated for viability before and after a heat shock of 50° for 6 min. The organisms were cooled in an ice bath after heating and were kept cold until plated. Viability was determined on minimal medium supplemented with 50 mg. of methionine and 20 mg. of yeast extract/l. and hardened by the addition of 1·5 % agar.

		Viable organisms (no. $\times 10^{-8}$)		
Pre-treatment of organisms	Incubation time in supplemented minimal medium (min.)	After washing in salts solution (*a*)	After washing in salts solution and heating 6 min. at 50° (*b*)	Ratio *a*/*b*:
None	0	4·8	0·32	15
	10	7·1	0·5	14
	30	3·9	0·44	8·9
Methylmethane-sulphonate (0·025 M)	0	0·81	0·00053	1530
	10	1·1	0·009	122
	30	1·2	0·12	10

as indicated by the inactivating effect of a 6 min. incubation in buffer at 50° and the differential heat sensitivity disappeared after the start of incubation in minimal medium (Spizizen, 1958) supplemented with 0·02 % acid casein hydrolysate and 50 μg./ml. each of L-tryptophan and L-methionine (Table 1). As the recipient strain for transformation we used the histidine + tyrosine + indole requiring triple mutant described by Nester & Lederberg (1961). This strain, supplied by Dr E. Nester, was used to permit the simultaneous determination of the effects of alkylation on linked markers. In one series of experiments utilizing DNA labelled with ^{32}P, wild type *Bacillus subtilis* was used as the DNA donor and the indole requiring strain (168) was used as recipient.

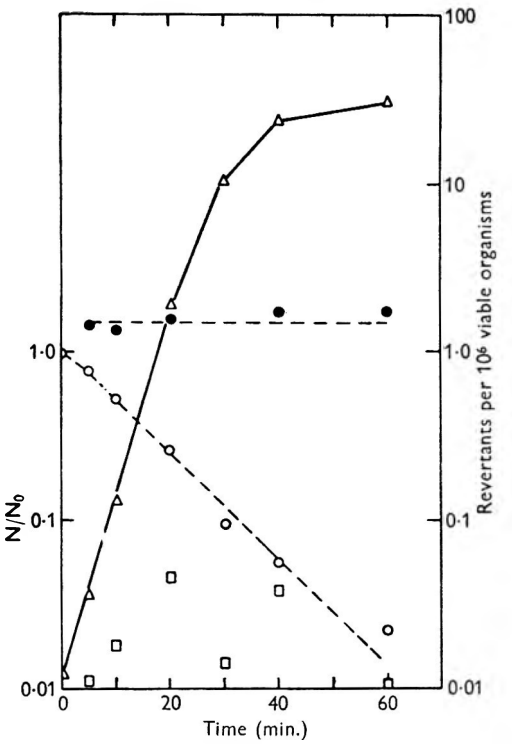

Fig. 1

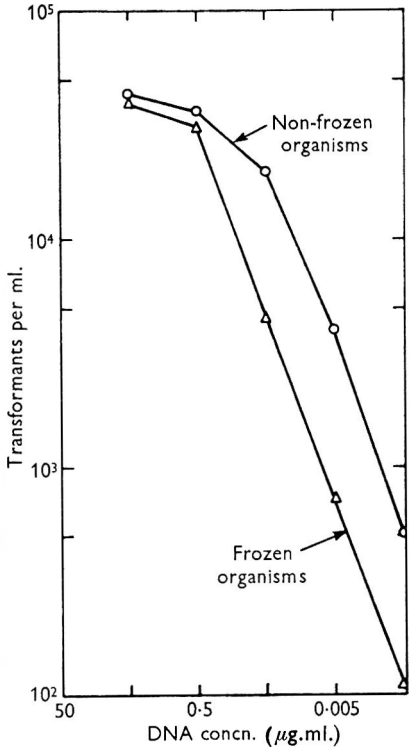

Fig. 2

Fig. 1 Effect of methylmethanesulphonate on the viability and reversion frequency of a methionine-requiring mutant of *Bacillus subtilis*. Broken line represents viability: circles indicate organisms treated with 0·025 M-methylmethanesulphonate; points represent non-treated control. Unbroken line, triangles represent reversion frequency of the treated samples; squares represent spontaneous mutation frequencies. Abscissa—time incubated with mutagen at 37°. An overnight culture of organisms was harvested, washed and suspended in 20 ml. salts solution. Ten ml. of suspension were added to 10 ml. of salts solution containing enough methylmethanesulphonate (0·043 ml.) to make the suspension 0·025 M. A second 10 ml. of suspension were added to 10 ml. of salts solution as a control. The resulting suspensions were incubated at 37° with occasional shaking. At the indicated intervals 2·5 ml. of suspension were removed and added to 7·5 ml. of cold salts suspension, centrifuged, washed once and made to 2·5 ml. with salts solution before plating. Revertants were determined on plates of minimal agar supplemented with 100 mg. yeast extract/l. Survivors were determined on plates of minimal agar (Spizizen, 1958) supplemented with yeast extract and 50 mg./l. methionine. Cell dilutions were made in salts solution to which 4 drops of 'Tween 80'/l. were added to prevent aggregation.

Fig. 2. Relation of DNA concentration to the number of transformants. DNA concentration is estimated from the absorption at 260 mμ of the purified preparation assuming an optical density of 200 for a 1 % (w/v) DNA solution. An overnight culture of the histidine + indole + tyrosine requiring triple mutant was used to inoculate 10 ml. of CHT 50 medium. After 4 hr. incubation at 37° with aeration the culture was harvested and diluted into 50 ml. of CHT 5 medium. Twenty-five ml. amouts of the suspension in CHT 5 were incubated 90 min. at 37° with shaking in Erlenmeyer flasks. Glycerol (2·5 ml.) was then added to one flask and the culture was quick frozen by immersion in a solid CO_2-acetone bath and stored at −25°. Non-frozen organisms (0·9 ml.) were then added to dilutions of standard DNA (0·1 ml.) and incubated 30 min. Deoxyribonuclease (5 μg./ml.) was added and after 5 min. the suspensions were plated for the determination of transformants at the indole locus. After this plating was concluded the frozen suspension was thawed by suspension with shaking for 15 min. in a water bath at 37° and this 'frozen' suspension was used as a recipient for transformation as described above.

Transformation was by slight modification of the method of Anagnostopoulos & Spizizen (1961). An overnight culture was inoculated into liquid minimal medium (Spizizen, 1958) containing 0·5% glucose, 0·02% acid hydrolysed casein and 50 μg. L-tryptophan/ml. (CHT 50 medium). After 4 hr. incubation with aeration at 37° the culture was harvested by centrifugation and diluted ten-fold in fresh minimal medium containing 0·5% glucose, 0·01% casein hydrolysate, 5 μg./ml. L-tryptophan and an additional 5 μM/ml. of $MgSO_4$ (CHT 5 medium). The organisms were incubated for 90 min. at 37° with shaking in 25 ml. of CHT 5 medium in a 250 ml. Erlenmeyer flask. Glycerol (2·5 ml.) was then added and the organisms were frozen by immersion of the flask in a solid CO_2-acetone mixture. Frozen organisms were stored at −25° for up to 10 days before use. During this storage period the competence of the frozen suspension gradually decreased to about one-tenth the original value for the frozen organisms. Frozen organisms were used for transformation as follows: the suspension was thawed by shaking for 15 min. in a water bath at 37° and then 0·9 ml. of suspension was added to a series of 150 × 16 mm. test tubes containing 0·1 ml. of the DNA solution to be assayed. The mixture of DNA plus organisms was shaken for 30 min. at 37° before addition of deoxyribonuclease (Worthington Biochemical Co.) to 5 μg./ml. The mixture was incubated with deoxyribonuclease for 5 min. at 37° before being plated for transformants. Transformation was scored on minimal medium (Spizizen, 1958) hardened with 1·5% agar and containing 20 mg. of Difco yeast extract/l. When only indole transformants were scored this medium was supplemented with 150 mg. casein hydrolysate/l. When histidine and tyrosine transformants were scored the minimal medium plus 20 mg. yeast extract/l. was supplemented with the proper combination of L-tryptophan (20 μg./ml.), L-tyrosine (20 μg./ml.) or DL-histidine (40 μg./ml.). Viable cell count was determined on minimal medium supplemented with 0·02% casein hydrolysate, 0·02% yeast extract and 20 μg. L-tryptophan/ml. Freezing decreased the amount of transformation obtainable from a given DNA preparation (Fig. 2) and the total percentage transformation was low (0·01–0·03) in our procedure using the triply auxotrophic mutant. However, the response to DNA was linear and reproducible.

Purified DNA for transformation experiments was prepared by the method of Marmur (1961) and was treated with alkylating agent *in vitro* by diluting the DNA in 0·1 M phosphate buffer (pH 7·0) containing either methyl- or ethyl-methanesulphonate to give a final concentration of DNA of 60 μg./ml. and allowing the reaction to proceed at 37°. Two volumes of 95% ethanol in water were then added and the suspension centrifuged after standing in the cold for 15 min. The precipitate was taken up in 0·015 M-sodium chloride + 0·0015 M-trisodium citrate (dilute saline + citrate; Marmur, 1961), the solution made 0·15 M-NaCl + 0·015 M-citrate (saline + citrate) and the DNA reprecipitated with ethanol and taken up in saline + citrate once again. This procedure was considered necessary to remove unreacted alkylating agent. DNA labelled with ^{32}P was prepared by growing wild type *Bacillus subtilis* in low phosphorus medium supplemented with $^{32}PO_4$ as described by Young & Spizizen (1961). The DNA was extracted from these organisms by the method of Marmur (1961).

In some of these experiments, organisms were treated with the mutagen and the DNA extracted from treated cells by a modification of the method of Stuy (1960)

who extracted DNA from an Haemophilus after X-ray irradiation. *Bacillus subtilis* was incubated overnight on slopes of a minimal salts medium (Spizizen, 1958) supplemented with yeast extract (0·15%), acid hydrolysed casein (0·1%), Difco nutrient broth (0·8%) and DL-tryptophan (40 mg./l.). This minimal salts medium had the following composition per litre of medium: $(NH_4)_2SO_4$, 2 g.; $MgSO_4.7H_2O$, 0·2 g.; K_2HPO_4, 14 g.; KH_2PO_4, 6 g.; $Na_3C_6H_5O_7.2H_2O$, 1 g.; glucose (sterilized separately), 5 g. This solution without glucose will be called 'salts solution' throughout this paper. Organisms were harvested by suspension in salts solution which was passed through a gauze filter to remove agar fragments. The organisms were washed and resuspended in salts solution and portions of the suspension were then added to salts solution in which sufficient methylmethanesulphonate was dissolved to make the final solution 0·025 M (0·085 ml./40 ml.). After reaction for 30 min. at 37°, the suspensions were harvested, suspended in 0·15 M-NaCl + 0·1 M-ethylene diaminetetra-acetate (pH 8; saline EDTA), centrifuged and suspended in a small volume of saline EDTA. Crystalline lysozyme was added to 1 mg./ml. and the suspension incubated for 30 min. at 37°. Sodium lauryl sulphate (0·5 ml. of a 25%, w/v, solution) was added, followed in 5 min. by sodium perchlorate to make the solution M. After deproteinization by shaking for 30 min. at room temperature with chloroform and isoamyl alcohol (24+1) the suspension was centrifuged for 10 min. at 5000 *g* and the upper layer transferred to a centrifuge tube. The tube was cooled and two volumes of cold 95% (v/v) ethanol added; after mixing and standing for 15 min. in the cold the suspension was centrifuged for 10 min. at 25,000 *g*, the supernatant poured off and the residue taken up in dilute saline citrate. The solution was made 0·15 M-NaCl + 0·015 M-citrate and clarified by centrifugation. The clarified supernatant was used as crude DNA extract.

DNA was determined by the diphenylamine reaction (Burton, 1956) with purified salmon sperm DNA as a standard. When small amounts of DNA were to be determined, 2 mg. bovine serum albumin were added as a carrier for the acid precipitate. Thymidine-2-^{14}C incorporation into organisms was determined on Millipore membrane filters as described by Kahan (1960). DNA was identified as material which could still be precipitated by cold perchloric acid after incubation for 1·5 hr. at 37° in N-NaOH but which was solubilized by treatment at 70° for 30 min. in 0·5 N-perchloric acid.

RESULTS

Purified DNA preparations were inactivated exponentially by heating at 50° after alkylation *in vitro* with either ethyl- or methyl-methanesulphonate (Fig. 3). Alkylation decreased the initial transforming activity and methylmethanesulphonate caused a greater inactivation on a molar basis than did the ethyl derivative. Inactivation of the linked indole and histidine markers occurred at the same rate on heating alkylated DNA. Labilization of DNA to heat after preliminary alkylation was first demonstrated by Zamenhof, Leidy, Hahn & Alexander (1956) who showed a complete inactivation of alkylated Haemophilus DNA after heating for 1 hr. at 76°. The decrease in activity of alkylated DNA and of DNA alkylated and heated for 5 hr. at 50° was not due to the failure of competent cells to take up this treated DNA (Table 2). DNA labelled with ^{32}P was fixed in a deoxyribonuclease-resistant form almost as efficiently after alkylation, and alkylation and heating as was control

labelled DNA in spite of the great difference in transforming efficiency of the treated and untreated preparations. In this experiment competent organisms of the indole-requiring strain (168) were prepared by the method of Young & Spizizen (1961) and used without preliminary freezing. Similar results showing that Pneumococcus transforming DNA inactivated by nitrogen mustard is taken up by competent cells have been obtained by Lerman & Tolmach (1959).

To determine whether alkylation and heating led to drastic degradation, the sedimentation coefficient of *Bacillus subtilis* DNA was determined in the Spinco

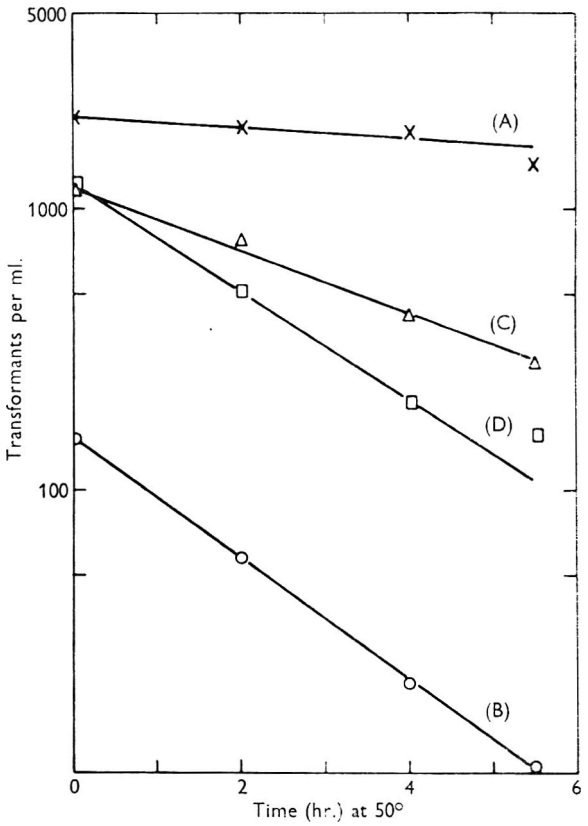

Fig. 3. Effect of heating at 50° on transforming activity. *A*, control, non-treated DNA: *B*, DNA which has been treated with 0·025 M-methylmethanesulphonate and freed from excess alkylating agent by precipitation twice with ethanol as described in the text; *C*, DNA treated with 0·1 M-ethylmethanesulphonate and treated as in *B*; *D*, DNA treated with 0·2 M-ethylmethanesulphonate and treated as in *B*. Four ml. of DNA at a concentration of 60 μg./ml. were reacted with alkylating agents for 1 hr. at 37° in 0·1 M-phosphate buffer (pH 7·0). After precipitation with ethanol and redissolving the precipitate in dilute saline + citrate the samples in 0·15 M-NaCl + 0·015 M-citrate were diluted 1:10 in salts solution before heating. Similar results were obtained when the heating at 50° was carried out in 0·1 M-phosphate buffer (pH 7·0).

Transformation at the indole locus was determined as described in the text using several dilutions of the treated DNA. The values for transformants were determined at DNA dilutions giving about 100 transformants per plate and were then corrected to the same dilution of DNA.

ultracentrifuge at a speed of 42,040 rev./min. with ultraviolet optics. The DNA was centrifuged in a solution made 0·15 M-NaCl + 0·015 M-citrate and the rotor temperature was kept below 8° to retard any further decomposition of alkylated DNA. The S_{20w} value of non-alkylated DNA was 26; DNA treated with 0·025 M-methyl methanesulphonate for 1 hr. at 37° gave an S_{20w} value of 25. After 6 hr. at 48° (a treatment which inactivated the DNA) the methylmethanesulphonate treated material had a S_{20w} value of 28.

When organisms were treated with 0·025 M-methyl methanesulphonate for 30 min.

Table 2. *Effect of alkylation and heating on the uptake of ^{32}P labelled* Bacillus subtilis *DNA*

Bacillus subtilis labelled with ^{32}P was prepared by growing wild type organisms on the medium described by Young & Spizizen (1961) and containing 5 mc. of $^{32}PO_4$ in 400 ml. of medium. DNA was prepared from these labelled cells by the method of Marmur (1961). The DNA was stored in saline citrate and after standing one day 76 % of the counts were precipitable with cold trichloroacetic acid plus carrier. The labelled DNA was alkylated as follows: 1 ml. of ^{32}P labelled DNA (optical density at 260 mμ = 2·14) was incubated for 90 min. with 1 ml. of 0·05 M-methylmethanesulphonate in 0·1 M phosphate buffer (pH 7·0). Two volumes of cold 95 % ethanol in water were added, the precipitate was collected by centrifugation and dissolved by adding 0·9 ml. of dilute saline citrate followed by 0·1 ml. of 1·5 M-sodium chloride + 0·15 M-sodium citrate. The resulting solution was diluted 1 to 6·25 and divided into two parts. One portion was heated for 5 hr. at 50° while the second was stored in the cold. Control DNA was taken through the same procedure with only the methylmethanesulphonate eliminated from the initial treatment.

Competent indole *Bacillus subtilis* organisms were prepared following the procedure of Anagnostopoulos & Spizizen (1961). After 90 min. incubation in CHT 5 medium (see text) this suspension was incubated with ^{32}P labelled DNA, 0·9 ml. of suspension was incubated with 0·1 ml. of the DNA solutions described above as well as with 1/10 and 1/100 DNA dilutions. At the conclusion of a 30 min. incubation period, 0·1 ml. of a 500 μg./ml. solution of crystalline deoxyribonuclease (Worthington Biochemical Co.) was added to the mixture of organisms and DNA. As a control separate portions of the DNA solutions were pretreated for 30 min. at 37° with deoxyribonuclease (plus 0·1 ml. of 0·1 M-$MgSO_4$) and then 0·9 ml. of organisms in CHT 5 medium (which was supplemented with extra Mg ion as described by Anagnostopoulos & Spizizen (1961)) was added. The difference between the counts fixed when deoxyribonuclease treatment of the DNA followed or preceded incubation of the organisms with DNA was taken as representing the DNA taken up. After incubation the organisms were either filtered through Millipore membrane filters, washed with salts solution and counted or used for the determination of transformants at the indole locus. The concentration of DNA was calculated from the optical density of its solutions at 260 mμ assuming that a 1 % DNA solution has an optical density of 200. The data are given for incubation of $1·16 \times 10^8$ viable organisms with 0·16 μg. of DNA labelled with ^{32}P ($6·06 \times 10^4$ c.p.m./μg. DNA) in 1·0 ml. of medium.

DNA sample	Transformed cells/ml.* at a DNA concentration of 0·16 μg./ml.	Deoxyribonuclease added: After incubation (c.p.m. fixed)	Deoxyribonuclease added: Before incubation (c.p.m. fixed)	Deoxyribonuclease added: Difference as μg. DNA	μg. DNA taken up/ transformed cell (no. $\times 10^6$)	μg. DNA taken up viable cell (no. $\times 10^{10}$)
Control	$6·97 \times 10^5$	1518	382	0·019	0·027	1·6
Control-heated	$5·51 \times 10^5$	1057	198	0·014	0·026	1·2
Methylated	$1·80 \times 10^5$	1626	310	0·022	0·121	1·9
Methylated-heated	$0·0537 \times 10^5$	1056	189	0·014	2·67	1·2

* Calculated from the numbers obtained at a DNA concentration of 0·0016 μg./ml. assuming linearity of response. The high values for transformants in this experiment are due to the use of fresh (unfrozen) cells of a highly competent indole strain.

at 37° (a treatment which decreased the fraction of viable organisms to 0·1, Fig. 1) the amount of transforming activity extractable was decreased as compared with the amount extracted from untreated organisms. In one experiment (Table 4) the transforming activity obtained from methylated organisms was 530 units (transformed colonies/μg. DNA) as compared to 6150 units for material from control organisms. The transforming DNA obtained from methylated organisms was inactivated by heating at 50° (Fig. 4).

Further purification of the extracts from treated and untreated organisms by continued chloroform+isoamyl alcohol deproteinization followed by a second ethanol precipitation yielded preparations with a ratio in specific activity, untreated: treated of 2·5 (data in Table 3). The more purified preparation of DNA from methylmethanesulphonate-treated organisms retained its heat sensitivity as compared to DNA from control organisms. The DNA from the treated organisms used in the experiment shown in Table 3 had a transforming activity 0·22 the original after 3 hr. heating at 50° whereas the DNA from the control culture was completely unaffected by the heating. Admixture of partially purified DNA prepared from methylated organisms did not sensitize untreated DNA to heat. There was a similar lack of sensitization when crude DNA preparations from treated and untreated organisms were mixed. These mixing experiments showed that extracts from alkylated organisms did not contain a substance which could produce a change in DNA resulting in heat sensitization as a secondary reaction. It should be recalled that even the crude preparations have been treated so as to inactivate any protein (enzymes) by denaturation. There was some evidence (Table 3) of competition between treated and untreated DNA at higher DNA concentrations since mixtures of DNA prepared from methylated and control organisms did not give the number of transformants expected on the basis of additivity at the higher DNA concentrations.

Crude preparations of DNA from methylmethanesulphonate-treated organisms did not wind around a glass rod when ethanol was added, although they were precipitated and collected by the centrifugation procedure. There was no significant difference in the amount of acid-precipitable diphenylamine-reacting material present in the crude DNA preparations from control and methylmethanesulphonate treated organisms, indicating that the DNA from alkylated organisms did not undergo differential breakdown to an acid soluble stage during isolation.

Effects of incubation in growth medium

Organisms were treated with methylmethanesulphonate and after washing out the mutagen the organisms were incubated in CHT 50 medium supplemented with L-methionine (50 μg./ml.). The organisms were incubated with rapid shaking at 37° in 250 ml. Erlenmeyer flasks containing 40 ml. medium. At intervals the organisms were harvested and washed with saline EDTA (pH 8). Crude DNA extracts were prepared and tested for transforming activity. As incubation proceeded the total amount of extractable transforming activity increased and the heat sensitivity of the extracted DNA diminished (Fig. 4).

The DNA content of the extracts and of the organisms from which the extracts were made did not show a comparable increase. For example, the DNA content of the organisms from which the extracts of Fig. 4 were made did not increase during the 150 min. incubation of this rather concentrated suspension. There was

an increase in the transforming units (trans rmed colonies/μg. DNA) in extract from alkylated organisms as compared with th :ontrols while incubation proceeded. In the experiment shown in Table 4 the DN: :xtracted from the methylmethane-sulphonate-treated organisms increased in act ity from 53 to 131 units, an increase

Table 3. *Competition between DNA extracted from methylated and control (untreated) organisms*

DNA was extracted from *Bacillus subtilis* treated for 30 min. at 37° with 0·025 M-methylmethanesulphonate in salts solution or incubated in salts solution as a control. The cells were washed, lysed with lysozyme as described in the text and deproteinized by treatment with sodium perchlorate and chloroform + isoamyl alcohol. After the initial precipitation with ethanol as described in the text the DNA preparation in saline-citrate was deproteinized by an additional three treatments with chloroform + isoamyl alcohol, reprecipitated with ethanol and dissolved in dilute saline citrate. The solution was made to 0·15 M-sodium chloride + 0·015 M-sodium citrate and stored in the cold. The transforming activity of the DNA from methylated organisms was decreased to 0·22 the original by heating for 3 hr. at 50°. The transforming activity of the DNA from control organisms retained all of its original activity after 3 hr. heating at 50°.

The predicted values are calculated from the number of transformants given by the extracts when tested separately. Transformation was scored at the indole locus. Each transformation tube had 0·1 ml. of the indicated DNA dilution added per ml.

Mixture	Dilution of mixture added to transformation tubes	Transformants/ml. Found	Predicted on basis of additivity
10·5 μg. of DNA from treated organisms per ml.	1	3600*	—
	1/10	610	—
	1/100	110	—
9·1 μg. of DNA from control organisms per ml.	1	7650*	—
	1/10	1850	—
	1/100	240	—
10·5 μg. DNA from treated organisms + 9·1 μg. DNA from control organisms per ml.	1	7050*	11,250
	1/10	2280	2460
	1/100	345	350
31·5 μg. DNA from treated organisms + 9·1 μg. DNA from control organisms per ml.	1	7300*	18,450
	1/10	2800	3680
	1/100	565	570

* These represent DNA concentrations in the non-linear transformation zone.

of about 150%, whereas the extracts obtained from control cultures altered by only 35%. The alkylated DNA lost its heat sensitivity as the incubation proceeded and approached the heat sensitivity of non-alkylated control DNA preparations.

During the period when the transforming units extracted from alkylated organisms increased from 530 to 1310 transformed colonies/μg. DNA in extract, the total cellular DNA in the treated culture increased by only 34% after an initial lag or slight decrease (Fig. 5). More of the total cellular DNA appeared to be extracted from the control non-alkylated organisms by the lysozyme procedure (Table 4). The percentage of DNA extracted was determined by comparison of the DNA values obtained by the analysis of whole organisms with values for DNA in the extracts. Analytical values for DNA in whole organisms are given in Fig. 5 while the concentration of DNA in the extracts is shown in Table 4. Although there was

no measurable increase in the total DNA in the cells during the first 80 min. incubation of methylmethanesulphonate-treated organisms (or even a slight decrease), new DNA was synthesized. Thymidine-2-^{14}C was linearly incorporated into alkylated organisms incubated in growth medium containing this substance (Fig. 5)

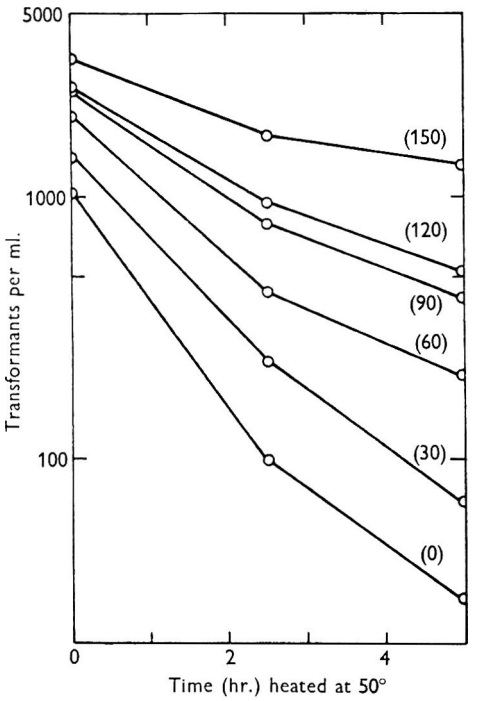

Fig. 4

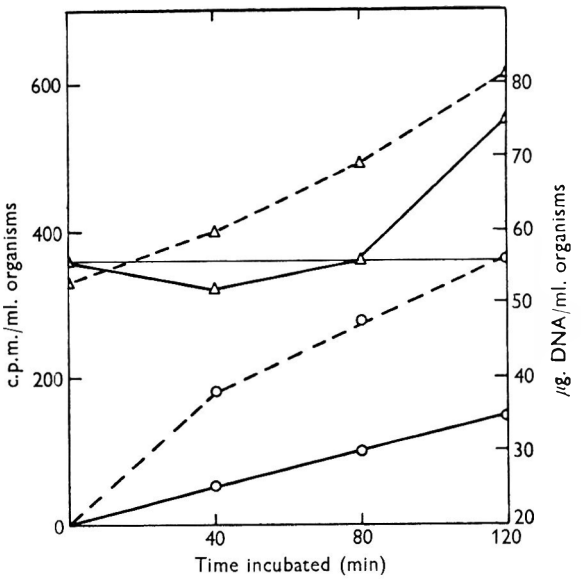

Fig. 5

Fig. 4. Effect of heat on the transforming activity of crude extracts of DNA prepared from alkylated organisms. The methionine-requiring organisms used were harvested after overnight culture at 37° from 16 slopes of the complete medium described in the text contained in 200 × 25 mm. screw cap tubes. After washing in salts solution the organisms were treated for 30 min. at 37° with 0·025 M-methylmethanesulphonate (0·085 ml./40 ml. of suspension in salts solution) and then washed with salts solution. A suspension of organisms in 12 ml. of salts solution was made and 2 ml. of this suspension were then added to 30 ml. of minimal medium supplemented with 0·02 % casein hydrolysate and 50 μg./ml. of L-methionine. The organisms were incubated with shaking at 37°. The times of incubation in min. are indicated in parenthesis on the figure. After incubation for the indicated time the organisms were harvested by centrifugation and washed with saline EDTA. A crude DNA preparation was then made from these cells as described in the text. The figures represent the number of transformants of the indole locus as determined by the standard assay procedure.

Fig. 5. DNA synthesis and thymidine-2-^{14}C uptake in alkylated organisms. Circles, thymidine uptake; triangles, DNA by diphenylamine reaction. Solid line, alkylated cells; broken line, controls. The thin line represents the amount of DNA present at the start of incubation. Two μc. thymidine (4×10^6 c.p.m.) of specific activity 10 μc./mg. were added to 40 ml. medium. After washing the organisms were taken up in 8 ml. saline EDTA for analysis. C.p.m. are recorded for material precipitated by perchloric acid after a suspension of organisms was incubated 90 min. at 37° in N-NaOH. The DNA determinations represent diphenylamine-reacting material extracted from whole cells by hot 0·5 M-perchloric acid as described in the text. Additional details of this experiment are given in the legend to Table 4.

and the incorporation began without lag from the start of incubation and proceeded in linear fashion. The incorporation was into the DNA since the radioactivity was solubilized by hot acid and was precipitated by cold acid even after 90 min. at 37° in N-NaOH. The absence of a lag in thymidine incorporation as contrasted to the long lag before the start of net DNA synthesis presents a paradox which will be considered in the discussion.

Table 4. *Effect of incubation on extractable transforming activity*

This table represents data from the same experiment shown in Fig. 5. The methionine-requiring organisms used were harvested after overnight culture at 37° from 24 slopes of the complete medium described in the text contained in 200 × 25 mm. screw cap tubes. The organisms were washed in salts solution and suspended in salts solution made 0·025 M with respect to methylmethanesulphonate (0·085 ml./40 ml. of salts solution). Control organisms were incubated in salts solution alone. After 30 min. incubation at 37° with gentle shaking the organisms were harvested, washed and resuspended in salts solution. Equal portions of this suspension of organisms were then incubated in 40 ml. minimal medium supplemented with casein hydrolysate (0·02 %), L-methionine (50 μg./ml.) and L-tryptophan (50 μg./ml.) and containing thymidine-2-^{14}C. The thymidine was added to give a concentration of 5 μg./ml. in a total volume of 40 ml. Each 40 ml. flask contained 2 μc. of thymidine-2-^{14}C. The organisms were incubated at 37° in a shaking water bath for the indicated time periods, washed with saline EDTA and then made to 8 ml. 4 ml. were used for the extraction of DNA while the remaining portion was used for the analysis of DNA and for the distribution of incorporated radioactivity. The values for transformants are from the linear portion of the response curve since tenfold dilution of the treated DNA gave a tenfold decrease in number of transformants. Transformation was scored at the indole locus.

Treatment	Time incubated in growth medium (min.)	DNA conc. of extract (μg./ml.)	% of all DNA extracted	DNA in assay tubes (μg.)	Transformants/0·1 ml.			Transformed colonies per μg. DNA (before heating)
					Non-heated	After 5 hr. at 50°	Ratio unheated: heated	
Methyl-methane-sulphonate	0	18·5	42	1·9	98	15	6·5	530
	40	14·5	35	1·5	167	18	9·3	1150
	80	16	36	1·6	188	44	4·3	1180
	120	18·5	31	1·9	241	90	2·7	1310
Control (untreated)	0	20	47	0·2	120	123	0·95	6150
	40	25·5	53	0·26	104	111	0·94	4080
	80	30·5	56	0·30	217	176	1·2	7100
	120	37·5	57	0·38	313	238	1·3	8300

DISCUSSION

The data can be analysed in terms of the following model. DNA is sensitized to heat by concentrations of methylmethanesulphonate which are lethal for the *Bacillus subtilis* used. The reaction of the cellular DNA with methylmethanesulphonate is assumed to occur in at least two main stages (Strauss, 1962*a*). The initial stage produces an alkylated DNA which participates in transformation and in replication (Green & Krieg, 1961). The second stage occurs after depurination of the alkylated DNA and may be assumed to be the formation of single strand breaks in the DNA. Treatment of the alkylated DNA at 50° accelerates the formation of single strand breaks.

The loss of activity at 50° in our experiments cannot be related to the thermal

denaturation of *Bacillus subtilis* DNA since heating the alkylated DNA for 5 hr. at 50° does not cause an increase in its absorption at 259 mμ (Strauss, 1962*b*) and since the T_m of *Bacillus subtilis* DNA in saline + citrate is 87·4° (Marmur, 1961). Alkylation of DNA does lead to a slight decrease in its thermal denaturation temperature (Strauss, 1962*b*) but the value (67·4° in 0·005 M buffer compared to 70·5° for control DNA) is still well above 50°. Furthermore, the lack of any major

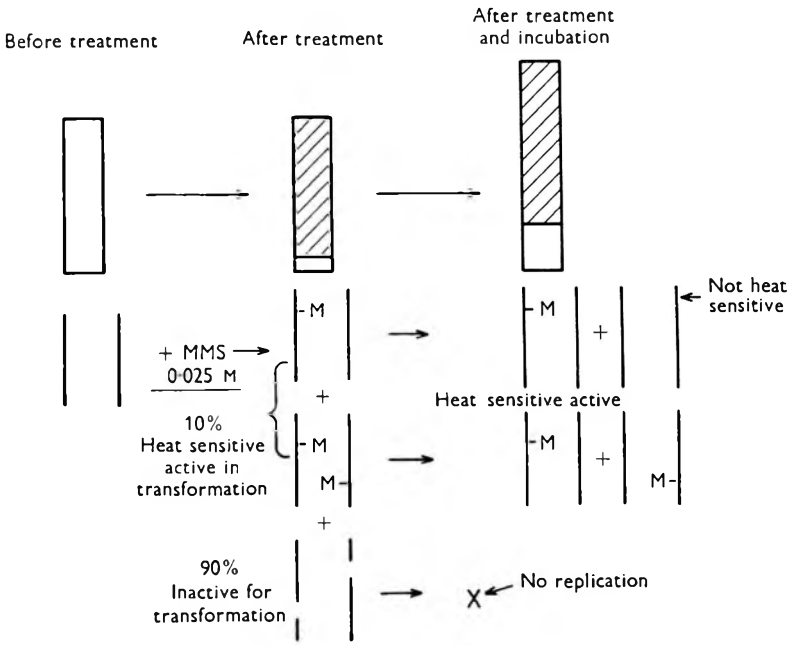

Fig. 6. Interpretation of the loss of heat sensitivity. The shaded areas represent DNA with single + double strand breaks which is supposed to be inactive for transformation of the indole locus and unable to replicate. Unshaded areas represent both alkylated and non-alkylated DNA able to replicate and of normal transforming activity. MMS = methylmethanesulphonate.

change in the sedimentation constant of alkylated or alkylated and heated *B. subtilis* DNA compared to non-treated material shows that the heating reaction does not cause any major degradation of the treated DNA into small molecules. Lerman & Tolmach (1957, 1959) showed that DNA with single strand breaks produced by deoxyribonuclease was inactive for transformation but was taken up by competent cells. Methylated, and methylated and heated *Bacillus subtilis* DNA is also taken up by competent cells almost as efficiently as untreated DNA (Table 2). The DNA extracted from alkylated organisms must consist of a mixture of alkylated DNA and of DNA with single-strand breaks, but there can be very little normal DNA in the extract. This follows from the heat sensitivity of extracts from alkylated organisms (Fig. 4); less than 3% of the original transforming activity remained after 5 hr. at 50°.

If this hypothesis be correct, then the heat sensitivity of DNA formed on an alkylated template will be determined by the average number of alkylated strands in the parental DNA (Fig. 6). If on the average only half of the strands of the marked (indole) region are alkylated, then replication will give a population in

which half the markers are heat sensitive and half are resistant. If both strands are alkylated, then replication will lead to no decrease in heat sensitivity, since it is supposed that a single-strand break can inactivate a marker and each marker will still contain one alkylated heat-labile strand. Further replication will of course produce heat-resistant markers (Fig. 6). This hypothesis may be used for a rough calculation of the degree of resistance to heat and the increase in transforming activity obtained on incubation of alkylated organisms in growth medium. The calculations are presented more to show that the hypothesis does not lead to absurd conclusions than for their quantitative validity. In the experiment shown in Table 4 and Fig. 5, the number of transformed colonies/μg. DNA obtained from the treated organisms increased from 530 to 1310 after incubation for 120 min. with a net increase of only 34% in the DNA. Heating for 5 hr. at 50° decreased the activity of the DNA from non-incubated organisms to 8·2 transformed colonies/μg. DNA. Consider an extent of methylmethanesulphonate reaction which leaves half of the progeny markers (marked regions in molecules) alkylated and heat sensitive (Fig. 6). The hypothesis requires that only the molecule active for transformation can participate in replication, and since the activity more than doubles I assume that all the active molecules participate in replication. For every 131 DNA molecules after replication there will then be $131/2 = 66$ heat sensitive and 66 non-sensitive molecules. If the ratio of activity before and after heating is the same as before incubation (i.e. 6·5) there will be $66/6{\cdot}5+66 = 76$ active molecules after heating, giving an unheated:heated ratio of $131:76 = 1{\cdot}7$. If some of the progeny DNA molecules were produced on a template of doubly alkylated DNA (Fig. 6) more of the progeny molecules would be heat sensitive. If only 1/4 of 131 molecules were resistant to heat after replication whereas the others still contained an alkyl group, as would occur if the templates for replication had been an equal mixture of doubly and singly alkylated molecules, there would be $33+98/6{\cdot}5 = 48$ biologically active molecules present after heating for 5 hr. at 50°, which would give a ratio of unheated : heated of $131:48 = 2{\cdot}7$, which was the ratio obtained (Table 4, line 4). (The situation is complicated by the fact that the 2·5-fold increase in transforming activity requires more than one round of replication for certain of the DNA molecules.) It is also clear that there can be an initial increase in transforming activity obtained from alkylated organisms upon incubation in growth medium without decreased heat sensitivity, if doubly alkylated molecules are used as templates.

The ratio of the transforming activity from treated and non-treated organisms was 530/6150 transformed colonies/μg. DNA = 0·086 (Table 4). It may be assumed that this is the fraction of the total DNA in treated organisms which is capable of serving as a template for replication, that is, that the DNA from treated organisms consists of 8·6% of alkylated but biologically active DNA and of 91·4% biologically inactive DNA with single+double strand breaks (Fig. 6). The active alkylated DNA would have the normal transforming activity which in this experiment was 615 transformed colonies/μg. DNA. Any new DNA synthesized would also have normal transforming activity. After incubation for 120 min. there was a 34% increase in the DNA of the treated material (Fig. 5). In every 1·34 μg. DNA extracted after incubation there would then be $0{\cdot}34+0{\cdot}086 = 0{\cdot}42$ μg. active DNA and 0·82 μg. inactive DNA which had not participated in replication. The active DNA would produce 6150 transformed colonies/μg. DNA so that after incubation

one would expect $0{\cdot}42 \times 615 = 258$ transformed colonies/$1{\cdot}34$ μg. or 193 transformed colonies/μg. DNA as compared with an actual figure of 131 transformed colonies/μg. after incubation and 53 transformed colonies/μg. DNA before incubation. If the newly synthesized DNA still containing an alkyl group had a lower transforming efficiency than normal DNA the calculated value would approach that found more closely.

The physical identification of the state produced by heating alkylated DNA at 50° with single strand breaks cannot be made with assurance in the absence of more physical data. It is still possible that this inactivation might represent the formation of apurinic sites, as suggested by the experiments of Brookes & Lawley (1961) and of Bautz & Freese (1960). It is also likely that the state of organisms after alkylation is more complex than we have indicated. Although the incorporation of thymidine-^{14}C by treated organisms was linear from the start of incubation, there was no net increase in the DNA of treated organisms until 120 min. of incubation. Actually a slight decrease in DNA content after the start of incubation was a regular feature of our experiments. It seems reasonable to suppose that some process occurs soon after the start of incubation which serves to degrade DNA that has been alkylated. A degradative process affecting alkylated DNA has been described (Strauss, 1962*b*). Breakdown of Haemophilus DNA was reported by Stuy (1960) after X-ray treatment. Degradation of DNA would of course introduce another element of uncertainty into the calculations above. Finally, there is no evidence that the 'recovery' from heat sensitivity of the extracted DNA (which actually represents synthesis of new DNA) is related to the rapid and dramatic recovery of viability of organisms from the effects of alkylation after very short periods of incubation in growth medium.

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Azurin: A Copper Protein Found in Bordetella

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SUMMARY

All strains of *Bordetella pertussis*, *B. bronchiseptica* and *B. parapertussis* tested, were found to contain a blue protein; this has been named 'Azurin'. It has been extracted from the cells of all three species and it has also been isolated from culture supernatant fluids of *B. pertussis*. The azurin was purified by chromatography on ion-exchange cellulose derivatives and obtained in physically pure form; the materials from each species were identical. In one strain of *B. bronchiseptica* azurin comprised 0·1 % of the bacterial dry weight. Azurin is a water-soluble autoxidizable protein of high oxidation-reduction potential (+395 mV.). It has an absorption maximum at 625 mμ in the oxidized form, with a millimolar extinction coefficient of $3{\cdot}5 \times 10^3$. It contains 0·45 % copper which can be completely removed by dialysis against cyanide. The molecular weight is 14,600 ($S_{20,w} = 1{\cdot}58 \times 10^{-13}$, $D_{20,w} = 10{\cdot}6 \times 10^{-7}$). Azurin can be reduced by cysteine, glutathione and other reducing agents; the blue colour disappears on reduction. Azurin undergoes reduction in the presence of concentrated cell-free extracts of *B. bronchiseptica* and succinate, and is reoxidized on aeration.

INTRODUCTION

Blue and green metallo-protein complexes have been isolated from a number of widely differing sources in nature. Several of the blue copper proteins isolated have been shown to be phenol oxidases, but other compounds without apparent enzymic activities have also been discovered. A green pigment, termed desulphoviridin, was obtained from the obligate anaerobic bacteria *Desulphovibrio desulphuricans* by Postgate (1956), while a copper protein was found in the chloroplasts of *Chlorella ellipsoidea* and in higher plants and named plastocyanin (Katoh, 1960; Katoh & Takamiya, 1961). A blue protein was first discovered in *Pseudomonas aeruginosa* some years ago (Verhoeven & Takeda, 1956); it was since isolated as a blue water-soluble, copper-protein complex by Horio (1958*a*) and its physical and chemical properties investigated (Horio, 1958*b*; Coval, Horio & Kamen, 1961). The presence of a blue protein in fractions obtained from *Bordetella pertussis* was first reported by Hammarsten, Palmstierna & Meyer (1959). The present paper describes the preparation, purification and some properties of a blue protein present in the three species of the genus *Bordetella*. A preliminary account of part of this work has appeared (Sutherland & Wilkinson, 1962).

METHODS

Organisms and methods of culture. The bacteria used were obtained from the National Collection of Type Cultures, Colindale, unless otherwise indicated. The strains used were: *Bordetella pertussis*, NCTC 8474, 4412 (obtained from Dr H.

Billaudelle, Karolinska Institute, Stockholm), 4507 (isolated at the City Hospital, Edinburgh), and 364 (the original Phase IV strain of Leslie & Gardner kindly provided by Dr Jean Dolby, Lister Institute, Elstree). Strain 364 was avirulent for mice and a further avirulent strain, 4507 AV, was obtained by repeated subculture of strain 4507. Two *Bordetella parapertussis* strains were NCTC 7385 and NCTC 8250. The *B. bronchiseptica* strains were NCTC 454, NCTC 8344, NCTC 8761 and NCTC 8762, strain BR/1 (isolated in the Bacteriology Department, Edinburgh University) and strain 8759 (obtained from Dr A. P. Maclennan, Microbiological Research Establishment, Porton).

Media. Virulent *Bordetella pertussis* strains were grown in the tris + resin liquid medium described in an earlier paper (Sutherland & Wilkinson, 1961). Other species were grown in a medium which contained (quantities/l. medium): Casamino acids (Difco technical) 4·0 g.; sodium glutamate, 4·0 g.; KH_2PO_4, 0·1 g.; $MgCl_2,6H_2O$, 0·08 g.; $FeSO_4,7H_2O$, 0·002 g.; $CuSO_4,5H_2O$, 0·004 g.; $CaCl_2$, 0·01 g.; yeast extract 200 ml. The yeast extract was prepared by the method of Cohen & Wheeler (1946). The medium was adjusted to pH 7·0 with N-NaOH, distributed in 250 ml. quantities into 500 ml. Ehrlenmeyer flasks and sterilized by autoclaving at 120° for 15 min. After inoculation the flasks were incubated at 37° and shaken at approximately 100 oscillations/min. on a reciprocating shaker (Distillers Company Ltd, Epsom). The cultures of *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* were incubated for 24, 48 and 72 hr., respectively. The bacteria were harvested by centrifugation at about 5000*g* for 30 min. in a MSE refrigerated centrifuge, and stored unwashed at −40° until required.

Absorption spectra. A Unicam SP 500 spectrophotometer with 1 cm. light path was used.

Total nitrogen. Nitrogen was determined by the micro-Kjeldahl method, the ammonia being trapped in boric acid and estimated colorimetrically with Nessler reagent.

Paper chromatography. Two-dimensional paper chromatography was used to identify the amino acids in protein hydrolysates. Whatman No. 1 paper sheets were run for 18 hr. with 80 % (w/v) phenol containing 0·3 % (v/v) ammonia. They were then dried, rotated through 90° and run for 24 hr. with butanol + acetic acid + water (4 + 1 + 5 by vol.). All chromatograms were run at 25°. Amino acid spots were detected with ninhydrin.

Buffer solutions. All salts used were analytical grade reagents. The pH values were read on a Pye 'Dynacap' pH meter.

Krebs–Ringer solution. This had the following composition (g./100 ml. solution): NaCl, 0·9; KCl, 0·04; $MgSO_4,7H_2O$, 0·04; $NaH_2PO_4,2H_2O$, 0·39; 0·4 ml. N-HCl.

Measurement of oxidation-reduction potentials. These were determined potentiometrically by the method of Gibson (1961) in an atmosphere of oxygen-free nitrogen.

Concentration of solutions of heat-labile substances. Bacterial extracts and other protein-containing solutions were concentrated by the method of Palmstierna (1960), by using a form of carboxymethyl cellulose known as 'courulose' (British Celanese Ltd, Coventry), Alternatively, polyethylene glycol of molecular weight about 6000 was used according to the method of Kohn (1959). Both methods were satisfactory, the first being preferable when it was necessary to concentrate large volumes.

RESULTS

Extraction of blue protein

Washings of the frozen *Bordetella pertussis* organisms in distilled water, or in Krebs–Ringer solution were blue in colour, but no coloured material was observed in washings from organisms of the other two species. This was probably because they were less easily lysed by the freezing and subsequent thawing. Several extraction procedures were investigated; the following routine was found to be applicable to all the species which were examined. The frozen bacteria (equivalent to about 50 g. dry weight, obtained from 30 l. of medium), were suspended in one l. Krebs–Ringer solution to which 0·1 M-urea had been added. The suspension was stirred at 0° for 30 min. by a high-speed stirrer, followed by centrifugation at *c.* 25,000*g* for 60 min. in the model L preparative Spinco ultracentrifuge. The supernatant fluid was stored in the cold and the bacterial deposit was extracted with a further 500 ml. of Krebs–Ringer solution containing urea. After centrifugation, as before, the supernatant fluid was again retained while the bacteria were suspended in 250 ml. distilled water, frozen and thawed. The viscous suspension was stirred as above, then centrifuged. The organisms were extracted with a further sample of distilled water, removed by centrifugation and discarded. The four extracts were pooled to give a viscous, opalescent solution; it was dark, greenish blue in colour. This solution was dialysed against cold running tap-water for 8 hr., then concentrated until the volume was about 100 ml. The extract was centrifuged at 100,000*g* for 15 min. in the ultracentrifuge. The deposit was washed with a small volume of distilled water, recentrifuged and discarded, the supernatant fluid being added to the initial solution. The coloured solution was dialysed against phosphate buffer (0·02 M-$KH_2PO_4 + Na_2HPO_4$) for 24 hr. at 4° with constant agitation. The non-diffusible material was applied to a column of DEAE cellulose equilibrated against the same buffer, and pumped through the column at a rate of 10 ml./hr. The column was then washed through with a further 20 ml. buffer, the washings being added to the original eluate. This was bright blue in colour and appeared to be considerably less viscous than the original solution. The blue eluate was concentrated and dialysed for 48 hr. against acetate buffer (0·02 M, pH 4·6). A variable amount of precipitate appeared at this stage and was removed by centrifugation at 100,000*g* for 15 min. The supernatant fluid was applied to a CM cellulose column in the sodium form, which had been thoroughly washed with the same acetate buffer. The coloured material was all adsorbed at the top of the column. The pH value was gradually increased, using a gradient of acetate buffer (0·02 M, pH 516) and 200 ml. mixer volume, and fractions were collected in an automatic fraction collector. The flow rate of the buffer was adjusted to 10 ml./hr. The blue material started to elute from the column at about pH 5·12. After the complete elution of the blue material, the top of the column was reddish brown in colour, probably due to the retention of cytochromes or other haemoproteins. The fractions containing blue material were concentrated, dialysed against acetate buffer (pH 4·6) and rechromatographed on CM Sephadex (Pharmacia, Uppsala) using the same conditions. The final product was concentrated, dialysed against glass-distilled water and either stored at −40° or lyophilized.

In typical experiments with *Bordetella bronchiseptica* strain 8344, equiv. 50 g.

dry weight of bacteria yielded 50–55 mg. of purified blue material. Although strains 454 and 8761 yielded comparable amounts of the product, the other strains and species contained less blue pigment. The differences did not appear to be due to incomplete extraction of the pigment from the bacteria, as ultrasonic disintegration of the cells at the end of the extraction process, followed by the complete extraction cycle, yielded no further blue material. In *B. pertussis* cultures, the longer incubation period resulted in some autolysis, with consequent liberation of blue pigment into the surrounding medium. When supernatants fluids from *B. pertussis* cultures were dialysed, concentrated and treated in the same way as cell extracts, about 0·2 mg. blue material/l. supernatant fluid was recovered. However, no pigment was isolated from the supernatant fluids of cultures of the other two species, even after prolonged incubation. This was probably because of the different degrees of autolysis occurring in these *Bordetella* species.

Properties of the blue protein

The blue material was present in all strains of the three Bordetella species tested. It could be extracted from the bacteria with Krebs–Ringer solution alone but omission of the urea made it necessary to increase the number of extractions to five or six before all the pigment was removed from the cells. The blue material could also be obtained in the soluble fraction after ultrasonic disintegration of the bacteria. All the pigment was in the solution after ultra-centrifugation of the sonic extracts and none was associated with the sedimentable portion.

The final product from ion exchange chromatography was very soluble in distilled water and solutions up to 10 % could be prepared. It was insoluble in chloroform, ether and ethanol.

Absorption spectrum. The absorption spectrum of the purified pigment from a pertussis strain is shown in Fig. 1. In the oxidized form, maxima were observed at 279 mμ and 625 mμ, but the latter peak disappeared on reduction. The millimolar extinction coefficient at 625 mμ, calculated on the basis of the molecular weight given below, was $3{\cdot}5 \times 10^3$. Table 1 gives the ratio of E_{625} to E_{280} at various stages of purification. Attempts to raise this value by other methods of purification, such as electrophoresis or chromatography on other ion exchange materials, were unsuccessful.

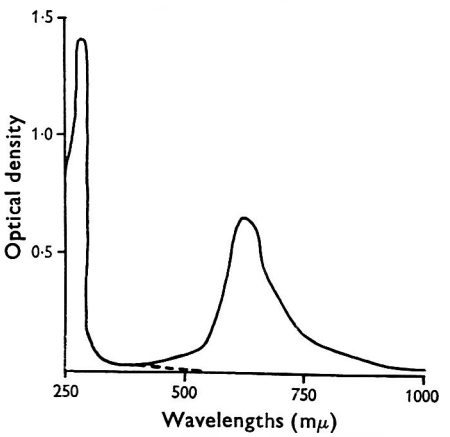

Fig. 1. Absorption spectrum of Bordetella blue protein

Stability. The blue material was readily oxidized by air or oxygen. In the oxidized form, no colour change was observed over the range pH 2·5–10·0. The material was rapidly destroyed by boiling, the colour disappearing and a white precipitate of denatured protein being deposited. The pigment could be lyophilized to a blue powder which was completely soluble in distilled water.

Oxidation-reduction potential. After purification, the pigment was in the fully oxidized blue form. It could be reduced by a variety of reducing agents such as ascorbic acid, cysteine, sodium thioglycollate or sodium dithionite. The oxidation-reduction potential of samples of the blue material (5 mg.) was titrated potentiometrically and found to be +395 mV at 20°.

Table 1. *Extinction ratios of blue protein at various stages of purification*

	$E_{625ox.}/E_{280}$
(1) Crude, pooled extract after concentration and ultracentrifugation	0·004
(2) Eluate from DEAE cellulose column	0·049
(3) As (2), after dialysis against acetate buffer (pH 4·6) and removal of the precipitate	0·108
(4) Eluate from CM cellulose column	0·343
(5) Eluate from CM Sephadex column	0·465

Nitrogen content. Determination of the total nitrogen in samples of the pigment extracted from different strains revealed a content of 14·9–15·3 %. This was indicative of a protein and paper chromatography of acid hydrolysates showed the presence of the following amino acids: cystine, glycine, aspartic acid, serine, glutamic acid, lysine, arginine, threonine, alanine, tyrosine, phenylalanine, isoleucine, leucine. The possible presence of others, in amounts too small to be detected by the techniques used, was not excluded.

Molecular weight. The sedimentation coefficient of the blue protein from *Bordetella bronchiseptica* was determined in a series of runs in a Spinco model E analytical ultracentrifuge. The samples, at three concentrations (0·15, 0·4 and 1·0 %), were dissolved in phosphate buffer (0·2 M, pH 7·0) and dialysed against the same buffer. The value obtained for the sedimentation coefficient extrapolated for zero protein concentration was $S_{20,w} = 1{\cdot}59 \times 10^{-13}$.

The diffusion coefficient was obtained from a single run for 36 hr. in a modified Tiselius electrophoresis apparatus, at 20°, from which the resultant $D_{20,w} = 10{\cdot}6 \times 10^{-7}$. The partial specific volume was assumed to be 0·75. This yields a molecular weight for the blue protein of 14,600 and a frictional ratio of 1·28. During sedimentation, a single peak was apparent and the blue peak moved with the Schlieren peak throughout the runs. Similar values were obtained for a run with a sample of *Bordetella pertussis* pigment.

Copper content. Spectrophotometric analysis indicated the presence of copper and the absence of iron, manganese and magnesium. Analysis of samples for their copper content was performed by activation analysis at the Atomic Energy Establishment, Wantage. The copper content of blue protein from four different strains is shown in Table 2; the mean value obtained from these results was 0·447 %. This, allied to the molecular weight, indicates the presence of one copper atom per

molecule of protein. No increase in copper content was obtained by dialysis against a copper sulphate solution followed by dialysis against distilled water to remove free copper ions. Dialysis against the chelating agent ethylenediamine tetra-acetic acid decreased the copper content of a sample of blue protein to 0·210%, while dialysis against cyanide completely removed the copper.

Table 2. *Copper content of blue protein from different Bordetella strains*

	Copper content (%)
B. pertussis 4412	0·436
B. pertussis 364	0·452
B. parapertussis 7385	0·448
B. bronchiseptica 8344	0·451

Metabolic activities. The blue protein was autoxidizable. A solution of the colourless reduced protein containing 100 μmole/ml. was completely oxidized in 20–25 min. at 37° when oxygen or air was passed through it. The rate of re-oxidation was very much greater, following the addition of small quantities of cell-free extracts of Bordetella species. The pigment was in the reduced form when the bacteria were harvested. This could be shown by visual examination of thick suspensions of *Bordetella bronchiseptica* cells immediately after harvesting; they were buff or pale pink in colour. However, when they were allowed to stand in air, or when air was passed through the suspensions, they rapidly turned greenish blue. This was seen most markedly in centrifuged bacterial deposits, the surface of which became blue while the underlying material remained pink. In the presence of succinate, the blue protein was rapidly reduced by cell-free extracts of Bordetella species.

Presence of similar pigments in other bacteria. Samples of bacteria of several other species were extracted by the method applied to Bordetella cells. Blue proteins, with properties very similar to those described for Bordetella material, were found in *Alcaligenes faecalis* NCTC 655, *Alcaligenes denitrificans* NCTC 8582 and *Pseudomonas fluorescens* strain 2. No trace of this type of protein was found in *Escherichia coli* B, *Salmonella typhimurium* strain 5, *Bacillus megaterium* strain 19, *Chromobacterium violaceum* NCTC 9757, *Achromobacter anitratus* NCTC 7844 or *Moraxella bovis* NCTC 8561.

DISCUSSION

The results described in this paper indicate that all three Bordetella species examined possess blue copper-proteins which are identical with respect to the tests that were made. We suggest the name azurin for this protein, as being in agreement with the nomenclature used for similar pigments, e.g. desulphoviridin (Postgate, 1956). The presence of copper-protein complexes of different function is widespread in nature. However, the only one of these compounds with any resemblance to azurin is the 'blue protein' isolated from *Pseudomonas aeruginosa* by Horio (1958*a*, *b*). This is remarkably similar in its redox potential (+390 mV), the amended molecular weight of 14,600 (Coval, Horio & Kamen, 1961) and in the presence of one atom of copper per molecule. Further, the 'blue protein' of *P. aeruginosa* can be purified by the method outlined for Bordetella azurin. A further similarity lies in the report that the Pseudomonas protein, like azurin, can be freed from copper by

dialysis against cyanide solution at neutral pH (Horio, Sekuzu, Higashi & Okunuki, 1961). In both cases, the copper could be restored by dialysis against copper sulphate solution. This property is common to several copper-protein complexes (Westerfeld, 1961). There is one difference in the reported properties; Horio (1958*a*) stated that the 'blue protein' was not autoxidizable. However, when it is prepared from *P. aeruginosa* by the method described above, the product is autoxidizable at a rate similar to that of azurin (Sutherland, unpublished results).

The continuous spectrum of azurin shows the absence of any Soret band similar to that found in desulphoviridin (Postgate, 1956). This indicates that the copper is not bound to the protein through a porphyrin molecule. The visible spectrum closely resembles that obtained for artificial copper-glycine complexes (Klotz, Faller & Urquart, 1950) and it seems possible that the chromophore is a copper-amino acid complex.

The function of azurin is uncertain. Its distribution appears to be somewhat limited in nature. The presence of azurin or similar pigments has only been found in the three genera *Bordetella*, *Alcaligenes* and *Pseudomonas*. No trace of similar compounds could be found in the species of *Salmonella*, *Escherichia*, *Achromobacter* or *Bacillus* tested. A relationship between the genus *Alcaligenes* and certain *Pseudomonas* species was indicated by Lysenko (1961) and it has been suggested that *A. faecalis* is related to *B. bronchiseptica* (Szturm & Bourdon, 1948). However, it is difficult to see what, if any, is the common metabolic process in azurin-containing micro-organisms. One important difference between the metabolism of *Pseudomonas* species and that of the genus *Bordetella* is the ability of the former to grow anaerobically. The conditions of growth used by Horio (1958*a*), 100 l. unaerated medium in deep vessels, would indicate that growth was anaerobic. It therefore appears that the 'blue protein' was produced under anaerobic conditions, although the actual yield was not reported. Nor is there any quantitative result for the production of the protein under different oxygen tensions. Azurin, however, is obtained from highly aerated cultures and it is remarkable that some strains of *B. bronchiseptica* contain as much as 0·1 % of their dry weight as this copper protein.

The 'blue protein' of *Pseudomonas aeruginosa* is thought to act as an alternative electron-transport system between a cytochrome of the *c* type (Pseudomonas cytochrome 551) and cytochrome oxidase (Horio, 1958*b*). Initial investigations with azurin, using *Bordetella bronchiseptica* cell-free extracts and purified cytochromes suggest a similar function (Sutherland, unpublished results).

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Changes in Gross Chemical Components of *Trichophyton mentagrophytes* During Incubation in Increased Carbon Dioxide Tensions

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SUMMARY

Macroconidia of *Trichophyton mentagrophytes* harvested from a culture grown on a medium rich in glucose contained 33% acid-soluble carbohydrate. Paper chromatography of hydrolysates showed glucose as the main sugar in this fraction; there was also a trace of an unidentified sugar. Macroconidia from the same strain grown on the same medium but deficient in glucose contained 19% carbohydrate; hydrolysates of this fraction contained glucose, with traces of galactose and the unidentified sugar. Microconidia harvested from a second strain grown on a medium rich in glucose contained 11% acid-soluble carbohydrate; hydrolysates of this fraction also contained glucose with traces of galactose and the unidentified sugar. These differences were reflected in whole cultures induced for macroconidial formation with carbon dioxide. Strains which responded well increased in acid-soluble carbohydrate; glucose was the predominating sugar in hydrolysates of these fractions. Strains which responded poorly did not increase in carbohydrate content; glucose, and traces of galactose and an unidentified sugar, were found in hydrolysates of these fractions.

INTRODUCTION

A previous report from this laboratory (Chin & Knight, 1957) described the stimulation of macroconidial formation in *Trichophyton mentagrophytes* during incubation at increased carbon dioxide tensions. A requirement for glucose was demonstrated, since macroconidia formation was not stimulated in increased atmospheric tensions of CO_2 when glucose was omitted from the medium. The experiments described in the present paper attempted to analyse and compare the gross chemical components of macroconidia, microconidia and hyphae. It was our intention to find some measurable difference between macroconidia and the other morphological units of *T. mentagrophytes* with the hope that this difference might be induced in the normally non-sporulating strains when macroconidia formation was stimulated by carbon dioxide. Because of difficulties involved in separating macroconidia from microconidia and hyphae, mutant strains which differed in type of sporulation were derived from a common parent. Macroconidia are easily harvested from a sporulating mutant which does not form microconidia, and vice versa.

METHODS

Mutants

All mutants were derived from *Trichophyton mentagrophytes* strain A-280 (from the Communicable Disease Center, Chamblee, Georgia, U.S.A.). Stock cultures were maintained by 3-monthly transfer on Sabouraud's glucose agar (Difco) and incubation at 30° for 10–14 days followed by storage at 5°. Sabouraud's glucose agar (4% glucose, 1% Neopeptone, 1·5% agar) was used throughout. Liquid medium was prepared by omitting the agar, and Sabouraud's conservation medium by the omission of glucose. Cultures were incubated for 5 days before harvest. The genetic purities of parent and mutant strains were assured by single spore isolations to avoid the selection of macroconidia-forming variants from mixed clones during incubation in CO_2 (Emmons & Hollaender, 1939). A microconidium was isolated from strain A-280 by the excision and transfer to fresh medium of a germinated microconidium from ruled agar blocks of Sabouraud's glucose agar. The clone resulting from the growth of this spore was maintained as the parent culture, strain 5-7. Spores from this clone were seeded on Petri plates containing solid media and irradiated with ultraviolet radiation. Morphological mutants differing in type of sporulation were selected from the survivors and transferred to fresh medium. In turn, clones resulting from the growth of transferred survivors were purified by the isolation of microconidia. Since strain 1-2-1 does not form microconidia, a macroconidium was isolated. The growth characteristics of all mutants have remained stable through 3 years of serial transfer. Their sporulating patterns and responses to CO_2 are recorded in Table 1.

Table 1. *Macroconidial formation by mutants of* Trichophyton mentagrophytes *and their response to increased carbon dioxide tensions in air*

Mutant	Normal air	16% (v/v) CO_2
5-7	rare	profuse
1-1-2	none	poor
1-2-1	abundant	profuse
1-3-1	none	poor

Inoculum and growth

Inoculum for solid media was prepared by dispersing the growth on a stock culture slope with sterile water and pipette. Cultures for chemical analysis were grown as surface mats on liquid media. A floating inoculum was prepared by pipetting 1 ml. medium on to the surface of a stock culture slope. The culture tube was rolled gently to dislodge spores and hyphal fragments, the majority of which floated on the surface of the medium. In inoculation, this preparation was poured into a flask containing liquid medium. Flasks which were incubated with increased atmospheric tensions of CO_2 (0–16% v/v) were fitted with rubber stoppers and glass tubing outlets. The gassing procedure was as previously described (Chin & Knight, 1957). After incubation the floating cultures were washed by pouring off the media and refloating the culture mats with water 4 times. The mats were then removed from the flasks and suspended in cold acetone (Umbreit, Burris & Stauffer, 1957). The organism was separated from the acetone by centrifugation and the supernatant acetone drawn off through a capillary pipette connected to an aspirator pump. Samples of organism were dried *in vacuo* overnight.

Spores

Spores were harvested from cultures grown on solid media in Roux bottles; macroconidia were harvested from strain 1-2-1, microconidia from strain 5-7. After 5 days of incubation, 40 ml. water were added to each bottle, the contents vigorously shaken and strained through gauze. Although many spores were retained with mycelium in the filter, the effluent usually contained a high percentage of the spores in the original harvest. Spores were separated from the effluent by centrifugation. The spores were washed 4 times, after which they were suspended in cold acetone and dried as described above.

Spore walls

Macroconidia were harvested and broken, prior to acetone treatment, in a Serval Omni-mix with water and glass microbeads for 30 min. The proportions of the mixture were spores + glass beads + water (1 + 2 + 3, by vol.). Microscopic examination of samples stained with lactophenol cotton blue revealed that 95% of the macroconidia were broken in all compartments. The spore walls were washed with water in excess of twenty times, suspended in cold acetone, and dried.

Extraction procedure

Weighed samples were transferred to test tubes and sequentially extracted in a boiling water bath with water for 15 min., with 5% (w/v) trichloroacetic acid (TCA) for 30 min., with N-NaOH for 30 min. The tubes were capped with glass marbles to prevent evaporation. After each extraction, the contents of the tubes were separated by centrifugation. Samples of extracts were removed for analysis with a pipetting bulb; the rest of the extract was discarded through a capillary pipette connected to an aspirator pump. The cell residues remaining after extraction with hot NaOH were washed with water four times by repeated centrifugation and transferred to tared aluminum cups. The residues were dried overnight at 80° and the weights determined by difference.

Chemical analyses

Carbohydrate was measured with the anthrone reagent, with glucose as the standard (Loewus, 1952). Samples from TCA extracts were hydrolysed with 0·6 N-HCl in a boiling water bath before paper chromatography. The solvent systems used for paper chromatography were isopropanol + water (8 + 2, by vol.) and amyl acetate + pyridine + water (3 + 3 + 1, by vol.). Paper chromatograms were developed with $AgNO_3$ reagent (Smith, 1958).

Protein was measured with the Folin phenol reagent method based on the modification of Lowry, Rosenbrough, Farr & Randall (1951).

Total nucleic acids (NA) were estimated by the method of Logan, Mannell & Rossiter (1952) at 268·5 mμ in a Beckman model DU spectrophotometer with 3·0 ml. silica cuvettes after extraction by the method of Schneider (1945). Samples were read against a blank of 5% TCA. Herring sperm deoxyribonucleic acid (DNA Nutritional Biochemical Co.), similarly hydrolysed, was used as the standard.

DNA content was determined by the method of Kech (1958). Ribonucleic acid (RNA) was estimated by the difference between total NA and DNA.

RESULTS

The gross chemical compositions of macroconidia, microconidia, and whole culture of *Trichophyton mentagrophytes* strain 5-7, which produces microconidia but no macroconidia on Sabouraud's glucose agar, are described in Table 2. The

Table 2. *Gross chemical components of macroconidia, microconidia, and whole culture of strain 5-7 of* Trichophyton mentagrophytes

Extract	Component	Macroconidia	Microconidia	Strain 5-7
		(% of dry wt.)		
H_2O	CHO*	11·3	13·2	7·6
	PRO	11·5	7·3	12·3
TCA	CHO	33·3	11·1	11·9
	PRO	5·5	5·4	5·8
	RNA	2·6	3·5	1·4
	DNA	0·2	0·3	0·1
NaOH	PRO	15·6	16·0	13·4
	CR	10·6	12·0	30·1

* CHO: carbohydrate, PRO, protein; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CR, cell residue.

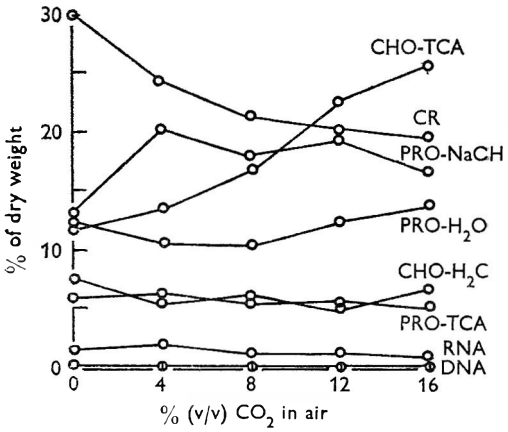

Fig. 1

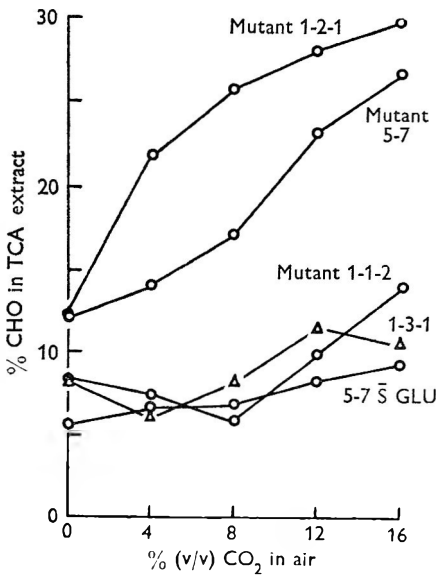

Fig. 2

Fig. 1. Changes in gross chemical composition, in percent of dry weight, of *Trichophyton mentagrophytes* strain 5-7 after incubation in increased carbon dioxide tensions. CHO-H_2O, hot-water-extractable carbohydrate; PRO-H_2O, hot-water-extractable protein; CHO-TCA, hot-TCA-extractable carbohydrate; PRO-TCA, hot-TCA-extractable protein; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PRO-NaOH, hot-NaOH-extractable protein; CR. cell residue.

Fig. 2. Changes in acid-soluble carbohydrate, in percent of dry weight, in mutants which vary in type and degree of sporulation during incubation in increasing carbon dioxide tensions. All mutants were harvested as surface cultures on liquid Sabouraud's medium, strain 5-7 was grown on medium with and without glucose (S̄ GLU).

macroconidia contained 33% hot-water-insoluble, hot-acid-soluble carbohydrate as compared with 11% in microconidia and 12% in strain 5-7. These differences became more striking when paper chromatograms of hydrolysates of these fractions were examined. Glucose and trace amounts of an, at present, unidentified sugar were found in the hydrolysate of the TCA extract from macroconidia; in microconidia and whole organisms of strain 5-7, glucose and trace amounts of galactose and the same unidentified sugar were found. The unidentified sugar is suspected to be fructose, rhamnose or sorbose. Macroconidia harvested from strain 1-2-1 grown on Sabouraud's conservation agar (prepared by the omission of glucose) contained 19% acid-soluble carbohydrate. Again, paper chromatography showed that

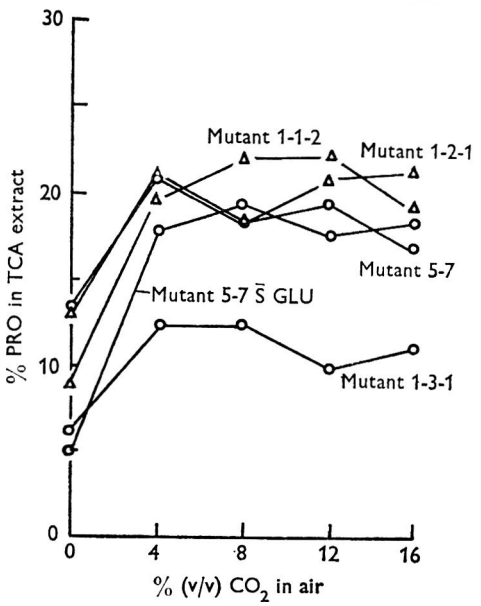

Fig. 3

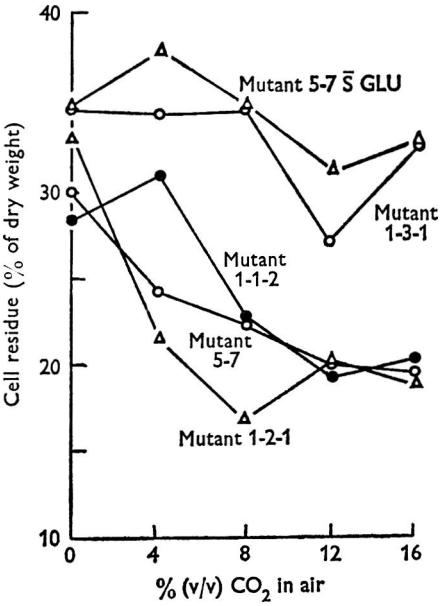

Fig. 4

Fig. 3. Changes in protein content, in percent of dry weight, in mutants which vary in type and degree of sporulation during incubation in increasing carbon dioxide tensions. All mutants were harvested as surface cultures on liquid Sabouraud's medium; strain 5-7 was grown on medium with and without glucose (S̄ GLU).

Fig. 4. Changes in cell residue, in percent of dry weight, in mutants which vary in type and degree of sporulation during incubation in increasing carbon dioxide tensions. All mutants were harvested as surface cultures on liquid Sabouraud's medium; strain 5-7 was grown on medium with and without glucose (S̄ GLU).

glucose was the predominant sugar in hydrolysates of this fraction; only traces of galactose and the unidentified sugar were found. Walls of macroconidia from strain 1-2-1 grown on Sabouraud's glucose agar contained 9% acid-soluble carbohydrate.

After pre-treatment with acetone, the cytoplasm of macroconidia, microconidia and hyphae stain red-brown with Lugol's iodine solution. Hyphal and spore walls do not take the stain.

Strains 5-7 and 1-2-1 responded markedly to incubation in increased atmospheric tensions of CO_2 and macroconidia formation was stimulated greatly. Strains 1-1-2

and 1-3-1 did not respond well to CO_2 and macroconidia formation was poorly stimulated. The changes in gross chemical components of the parent strain, 5-7, grown in increasing amounts of CO_2 are shown in Fig. 1. The greatest fluctuations occur in three fractions: acid soluble carbohydrate (CHO-TCA), NaOH-extractable protein (PRO-NaOH), and cell residue (CR). The changes in these fractions, for all mutants, in response to CO_2 are described in Fig. 2 (CHO-TCA), Fig. 3 (PRO-NaOH), and Fig. 4 (CR). In strains 5-7 and 1-2-1, increases in carbohydrate and protein, and decreases in cell residue, accompanied increased macroconidia formation as it was induced by increasing CO_2 tensions. Strains 1-1-2 and 1-3-1 were poorly stimulated to form macroconidia and did not show increases in carbohydrate. Under increased atmospheric tensions of CO_2, strain 1-1-2 increased in protein and decreased in cell residue; strain 1-3-1 did not increase in protein or decrease in cell residue. Paper chromatograms of TCA extract hydrolysates from all cultures showed that glucose was the predominant sugar in these fractions; traces of galactose and the unidentified sugar were also present. When strain 5-7 was grown on Sabouraud's conservation medium under increased tensions of CO_2, macroconidia formation was poorly stimulated. Analyses of gross chemical components during incubation with CO_2 revealed no increase in carbohydrate, an increase in protein, and no decrease in cell residue.

DISCUSSION

The formation of macroconidia and microconidia by *Trichophyton mentagrophytes* implies that differences in structure and function, as well as size, exist between the two spore forms. Unfortunately, information about functional differences between macroconidia and microconidia is not available. One difference in function has been implied (Wilhelm, 1947). Since macroconidia are multinucleate and microconidia are uninucleate (Emmons, 1934), a heterokaryotic state may exist in the multinuclear compartment of a macroconidium and provide a survival advantage by allowing the macroconidium to carry a recessive gene.

The stimulation of macroconidia formation by incubation in increased CO_2 tensions is difficult to investigate, since very little is known about the physiology of the organism. If any existing differences in the gross chemical components of morphological structures were known, it should be possible to investigate the physiological changes in mycelia during the process of sporulation and the effect of CO_2 on these changes. The experiments described in this paper were designed to this end.

It was previously demonstrated that stimulation of macroconidia formation by incubation in increased CO_2 tensions did not occur when glucose was omitted from the medium. This requirement for glucose may be a direct reflexion of the higher concentration of carbohydrate in macroconidia than in microconidia or hyphae. Perhaps the formation of macroconidia requires higher concentrations of glucose in the medium for a structural precursor of the spore itself. The carbohydrate appears to reside in the cytoplasm.

It is our experience that macroconidia germinate more rapidly than microconidia on Sabouraud's glucose agar. Wheeler, Cabaniss & Cawley (1958) showed that macroconidia of *Microsporum fulvum*, another dermatophyte, were capable of germinating in distilled water in 4·5–10 hr. (confirmed in our laboratory). Macroconidia of

Trichophyton mentagrophytes germinate in distilled water in 8 hr. when they are aerated; microconidia will not germinate under the same conditions. We have postulated that the higher concentration of carbohydrate in macroconidia may be more available as an energy source for germination than the lower quantity in microconidia. This is not the only factor to be considered, however, since microconidia suspended in 4% glucose solution will not germinate in 48 hr.

Other changes in gross chemical components of cultures also occur during incubation in increased CO_2 tensions, particularly, in protein and cell residue fractions. However, investigations into these two fractions have been deferred because the separation of strong macroconidia-forming mutants and weak macroconidia-forming mutants is not as clear as in the carbohydrate fraction. These fractions have not, by any means, been dismissed from further consideration since the process of macroconidia formation must be complex. Physiological and structural changes during sporulation will rely heavily upon the formation of new and more protein. Generalizations about the sporulating processes of fungi are difficult to make. The factors which influence spore formation are many and varied (Cochrane, 1958); an agent which stimulates sporulation in one organism may inhibit it in another. Comparisons of sporulating processes will have to await more studies similar to those of Wright & Anderson (1958) on *Dictyostelium discoideum* and Cantino (1956) on *Blastocladiella emersonii*. It is of interest that *D. discoideum* accumulates cellulose during one phase of sporulation and that CO_2 induces the formation of resistant sporangia in *B. emersonii*. Cantino & Horenstein (1956) implicated a succinate-ketoglutarate-isocitrate cycle in the sporulation of Blastocladiella. Whether these aspects of carbohydrate metabolism are related to the accumulation of glucose in *Trichophyton mentagrophytes* remains to be seen.

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Stimulation of Glucose Metabolism in *Trichophyton mentagrophytes* during Incubation in Increased Carbon Dioxide Tension

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SUMMARY

An inducible UDPG pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) has been demonstrated in cell-free extracts from *Trichophyton mentagrophytes* incubated in increasing atmospheric tensions of carbon dioxide. Assays of related enzymes have shown that UDPG pyrophosphorylase is not the only enzyme induced by CO_2. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glucose-phosphate isomerase (EC 5.3.1.7) were more strongly stimulated than UDPG pyrophosphorylase. Hexokinase (EC 2.7.1.1), phosphoglucomutase (EC 2.7.5.1) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) were more weakly stimulated than UDPG pyrophosphorylase. The stimulation of UDPG pyrophosphorylase does not occur as a result of sequential induction.

INTRODUCTION

Two previous papers from this laboratory (Chin & Knight, 1957, 1963) described the stimulation of macroconidial formation in *Trichophyton mentagrophytes* when the organism was grown on Sabouraud's glucose medium and incubated under increasing atmospheric tensions of CO_2. When glucose was omitted from the medium, macroconidial formation was not stimulated. Macroconidia contain 33% acid-soluble carbohydrate; microconidia and the whole culture of a strain, which produces microconidia but no macroconidia, contain 11% and 12% carbohydrate, respectively. A correlation between increase of acid-soluble carbohydrate and macroconidial formation was demonstrated in whole cultures as they were incubated in increased CO_2 tensions. Two strains, 5-7 and 1-2-1, were strongly stimulated to form macroconidia and increase in carbohydrate content; two strains, 1-1-2 and 1-3-1, were weakly stimulated to form macroconidia and did not increase in carbohydrate content.

A preliminary experiment, which forms the basis of this paper, demonstrated the stimulation of uridinediphosphoglucose (UDPG) pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) activity in cell-free extracts of cultures incubated under increased CO_2 tensions. The study of polysaccharide formation during induction of macroconidial formation by incubation of the organism in increased CO_2 tensions was divided into three parts: (1) the effect of CO_2 on other enzymes involved in polysaccharide formation; (2) the chemical nature of the polysaccharide; (3) an attempt to demonstrate the transfer of glucose from UDPG to polysaccharide. The first part of these studies is described in this paper.

METHODS

Mutants and culture methods. The mutants and culture methods used were as previously described (Chin & Knight, 1957, 1963). *Trichophyton mentagrophytes* strains 5-7 and 12-1 which are strongly stimulated to form macroconidia and increase in carbohydrate content during incubation in increased CO_2 tensions, as well as strains 1-1-2 and 1-3-1 which are weakly stimulated to form macroconidia and do not increase in carbohydrate content, were used in the present work. Cultures were grown as surface mats on Sabouraud's liquid medium under increasing tensions of CO_2 (normal air to 16%, v/v). The inoculating and gassing procedures were as described previously.

Cell-free extracts. After 4 days of incubation at 30°, floating cultures were washed by pouring off the medium and refloating the culture mats with water 4 times. The mycelial mats were transferred to chilled mortars, and after cold 0·02 M-sodium acetate buffer (pH 5·9) and acid-washed sand were added, the mats were crushed with a chilled pestle for 5 min. The crushed material and sand was transferred to test tubes and the supernatant solutions separated by centrifugation at 700 *g* for 5 min. The supernatant solutions, which will be referred to as cell-free extracts, were withdrawn with a pipetting bulb.

Protein determination. The protein content of cell-free extracts was measured by the method of Lowry, Rosenbrough, Farr & Randall (1951) with bovine serum albumin as the standard.

Assays of enzymes. Enzymatic activities of cell-free extracts were measured in a Beckman Model DK-II Recording Spectrophotometer with a thermostatically controlled (30°) cuvette holder and 1 ml. silica cuvettes. All enzymes, with the exception of UDPG pyrophosphorylase, were assayed by taking advantage of an active glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in cell-free extracts and measured by the reduction of nicotinamide-adenine dinucleotide phosphate (NADP) at 340 mμ. Cell-free extract equivalent to 100 μg. protein was used in each assay. However, where activities were low, notably in the assay of hexokinase (EC 2.7.1.1), up to 500 μg. protein was used. Specific activities are expressed as the increase in optical density at 340 mμ/mg. protein/min.

Hexokinase (*EC* 2.7.1.1). The hexokinase assay was adapted from the method of Chattaway, Thompson & Barlow (1960), using a volume of 1 ml. The reaction mixture contained 30 μmoles 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7·4), 0·25 μmole glucose, 0·06 μmole $MgSO_4$, cell-free extract equivalent to 100 μg. protein, 0·50 μmole NADP and 1·00 μmole adenosine triphosphate (ATP). ATP was omitted from the reference cuvette and added to the sample cuvette to start the reaction.

Glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49). Glucose-6-phosphate dehydrogenase activity was assayed by an adaptation of the phosphohexoseisomerase assay by Slein (1955). The reaction mixture contained 30 μmoles tris buffer (pH 7·4), 0·25 μmole glucose-6-phosphate, 1·00 μmole $MgCl_2$, 0·50 μmole NADP and cell-free extract equivalent to 100 μg. protein in a total reaction volume of 1 ml. Glucose-6-phosphate was omitted from the reference cuvette and added to the sample cuvette to start the reaction.

Glucosephosphate isomerase (*EC* 5.3.1.9). Glucosephosphate isomerase was

assayed by the method of Slein (1955) in a total reaction volume of 1 ml. The reaction mixture contained 30 μmoles tris buffer (pH 7·4), 0·25 μmole fructose-6-phosphate, 1·00 μmole $MgCl_2$, 0·50 μmole NADP, and cell-free extract equivalent to 100 μg. protein. Fructose-6-phosphate was omitted from the reference cuvette and added to the sample cuvette to start the reaction.

6-Phosphogluconate dehydrogenase (*EC* 1.1.1.43). 6-Phosphogluconate dehydrogenase was assayed after adapting the method of Chattaway *et al.* (1960) to a reaction volume of 1 ml. The reaction mixture contained 30 μmole tris buffer (pH 7·4), 0·25 μmole 6-phosphogluconate, 0·60 μmole $MnCl_2$, 0·50 μmole NADP, and cell-free extract equivalent to 100 μg. protein. 6-Phosphogluconate was omitted from the reference cuvette and added to the sample cuvette to start the reaction.

Phosphoglucomutase (*EC* 2.7.5.1). Phosphoglucomutase was assayed after adapting the method of Chattaway *et al.* (1960) to a reaction volume of 1 ml. The reaction mixture contained 30 μmoles tris buffer (pH 7·4), 0·25 μmole glucose-1-phosphate, 0·60 μmole $MnCl_2$, 0·50 μmole NADP, and cell-free extract equivalent to 100 μg. protein. Glucose-1-phosphate was omitted from the reference cuvette and added to the sample cuvette to start the reaction.

UDPG pyrophosphorylase (*EC* 2.7.7.9). UDPG pyrophosphorylase was assayed by a modification of the method of Kalckar & Anderson (1957). The reaction mixture contained, in a volume of 1 ml., 100 μmoles glycine buffer (pH 8·7), 0·1 μmole uridine triphosphate (UTP), 0·6 μmole glucose-1-phosphate (G-1-P), 1·0 μmole $MgCl_2$, 1·0 μmole NAD, cell-free extract equivalent to 100 μg. protein, and 5 units commercial UDPG dehydrogenase (Sigma Chemical Co.). Two reactions were run simultaneously: the formation of UDPG from UTP and G-1-P by UDPG pyrophosphorylase and the oxidation-reduction of UDPG to UDP-glucuronic acid and NAD to $NADH_2$ by UDPG dehydrogenase (EC 1.1.1.22). Under the conditions described, UDPG pyrophosphorylase was maintained as the rate-limiting reaction. UTP was omitted from the reference cuvette and added to the sample cuvette to start the reaction. Progress of the reaction was measured by the reduction of NAD at 340 mμ.

RESULTS AND DISCUSSION

The demonstration of an inducible UDPG pyrophosphorylase in *Trichophyton mentagrophytes* during incubation in increasing carbon dioxide tensions is presented as presumptive evidence that the concomitant accumulation of high levels of polysaccharide proceeds via this pathway rather than directly through glucose-1-phosphate directly (Sols, 1961). The experiments in this paper were designed to determine whether UDPG pyrophosphorylase was the only enzyme, in the early steps of glucose metabolism, which was stimulated by CO_2, or, if other enzymes are stimulated, whether the induction of UDPG pyrophosphorylase is sequential (Stanier, 1950). Four strains of *T. mentagrophytes*, derived from the same parent, were used. Two strains, 5-7 and 1-2-1, responded strongly to incubation in increased CO_2 tensions; macroconidial formation was profuse and high levels of polysaccharide accumulated. Two strains, 1-1-2 and 1-3-1, responded weakly to incubation in increased CO_2 tensions; macroconidial formation was poor and high levels of polysaccharide were not accumulated. Cell-free extracts from cultures incubated in increasing CO_2 tensions were assayed for hexokinase, phosphoglucomutase, UDPG

pyrophosphorylase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and glucosephosphate isomerase activities. The results of these assays, as they are depicted in Figs. 1, 2, 3 and 4, demonstrate that UDPG pyrophosphorylase was not the only enzyme which was induced by CO_2. Glucose-6-phosphate dehydrogenase and glucosephosphate isomerase were more strongly stimulated than UDPG pyrophosphorylase. Hexokinase, phosphoglucomutase and 6-phosphogluconate dehydrogenase were more weakly stimulated, if at all, than UDPG pyrophosphorylase. Sequential induction of UDPG pyrophosphorylase, through stepwise induction of hexokinase and then phosphoglucomutase before induction of UDPG pyrophosphorylase, was not observed.

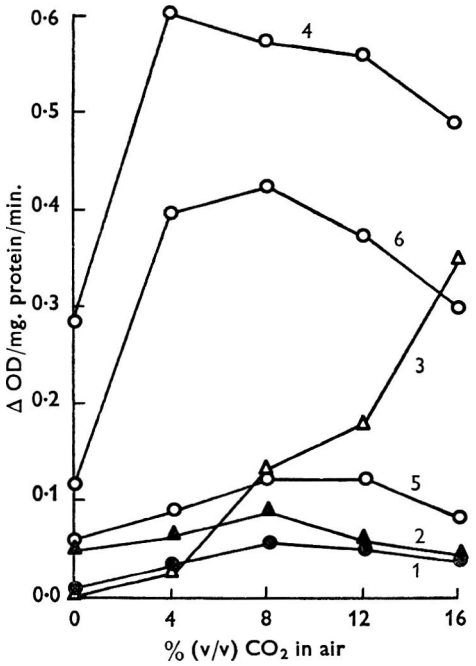

Fig. 1

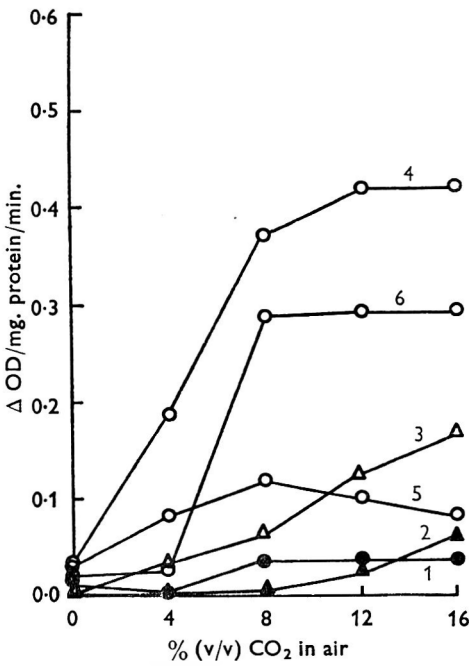

Fig. 2

Fig. 1. Changes in specific activities, as ΔOD/mg. protein/min., of enzymes in cell-free extracts from strain 1-2-1 grown in increased carbon dioxide tensions. The enzymes assayed were (1) hexokinase, (2) phosphoglucomutase, (3) UDPG pyrophosphorylase, (4) glucose-6-phosphate dehydrogenase, (5) 6-phosphogluconate dehydrogenase and (6) glucosephosphate isomerase.

Fig. 2. Changes in specific activities, as ΔOD/mg. protein/min., of enzymes in cell-free extracts from strain 5-7 grown in increased carbon dioxide tensions. The enzymes assayed were (1) hexokinase, (2) phosphoglucomutase, (3) UDPG pyrophosphorylase, (4) glucose-6-phosphate dehydrogenase, (5) 6-phosphogluconate dehydrogenase, and (6) glucosephosphate isomerase.

On the other hand, conditions which contribute to the accumulation of polysaccharide during incubation in increased CO_2 tensions were shown to exist. The greatest amount of enzyme stimulation resided in glucose-6-phosphate dehydrogenase, glucosephosphate isomerase and UDPG pyrophosphorylase. Figures 1 and 2 show that UDPG pyrophosphorylase activity in strains 5-7 and 1-2-1, which were

strongly stimulated by CO_2 to form macroconidia and accumulate polysaccharide, continued to increase after glucose-6-phosphate dehydrogenase, the first enzyme leading into the hexosemonophosphate shunt, and glucosephosphate isomerase, the first enzyme leading into glycolysis, had attained maximum stimulation. In strain 1-3-1, which was weakly stimulated by CO_2 to form macroconidia and accumulate polysaccharide, the stimulation of UDPG pyrophosphorylase activity followed a course similar to those of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase activities. These three enzymes were stimulated (Fig. 3) to their maxima at 8% (v/v) CO_2. Figure 4 shows that the stimulation of all enzymes in strain 1-1-2, which responded poorly to CO_2, was weaker than in the other strains tested.

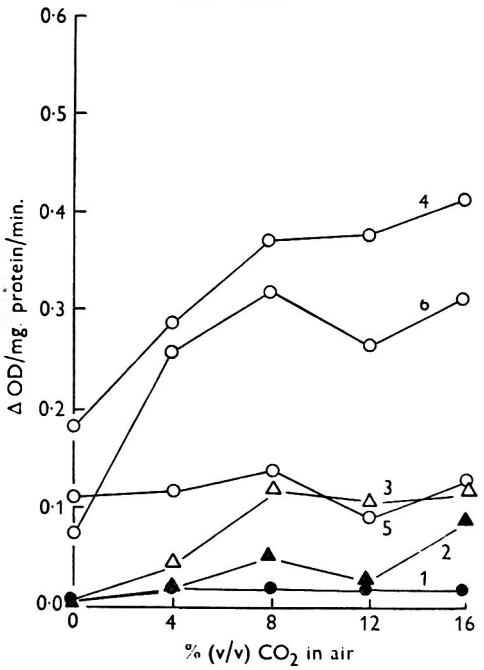

Fig. 3

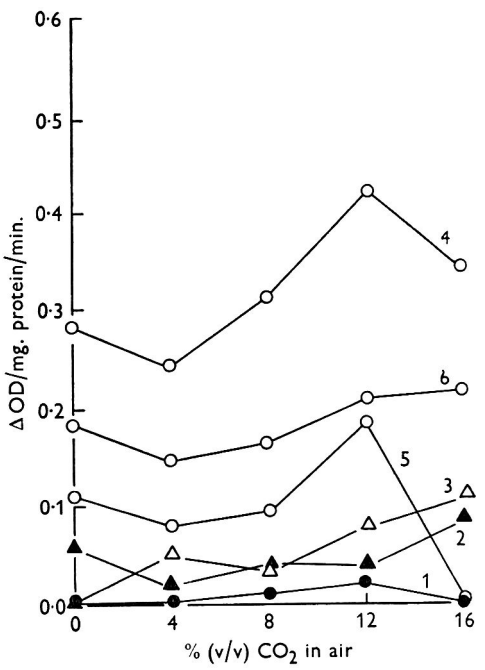

Fig. 4

Fig. 3. Changes in specific activities, as ΔOD/mg. protein/min., of enzymes in cell-free extracts from strain 1-3-1 grown in increased carbon dioxide tensions. The enzymes assayed were (1) hexokinase, (2) phosphoglucomutase, (3) UDPG pyrophosphorylase, (4) glucose-6-phosphate dehydrogenase, (5) 6-phosphogluconate dehydrogenase, and (6) glucosephosphate isomerase.

Fig. 4. Changes in specific activities, as ΔOD/mg. protein/min., of enzymes in cell-free extracts from strain 1-2-1 grown in increased carbon dioxide tensions. The enzymes assayed were (1) hexokinase, (2) phosphoglucomutase, (3) UDPG pyrophosphorylase, (4) glucose-6-phosphate dehydrogenase, (5) 6-phosphogluconate dehydrogenase and (6) glucosephosphate isomerase.

These results pose two questions which must be answered as the physiological study of *Trichophyton mentagrophytes* is continued. What is the mechanism by which CO_2 induces glucose-6-phosphate dehydrogenase, glucosephosphate isomerase and UDPG pyrophosphorylase? Does the stimulation of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase imply that CO_2 may bring about

macroconidia formation by stimulating the hexosemonophosphate shunt and/or glycolysis?

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Autolytic Enzymes as a Source of Error in the Preparation and Study of Gram-negative Cell Walls

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SUMMARY

The cell wall of *Salmonella gallinarum*, like that of other Gram-negative organisms, was found to contain a separate continuous rigid mucopolymer layer. This structure was rapidly broken down by the cell's own enzymes during wall preparation when suitable precaution against this action was not taken. Possible errors in the interpretation of cell-wall structure, deriving from this and other causes, are discussed.

INTRODUCTION

Recent work on Gram-negative cell-wall structure has led several authors, working independently, to differing views about the mucopolymer component of the wall (cf. Shafa & Salton, 1960; Work, 1961; Weidel, Frank & Martin, 1960). The opposing views are that the wall contains no continuous separate mucopolymer layer or that it does contain such a layer (*R*-layer). Its absence was concluded from two sets of observations. Shafa & Salton (1960) found complete disaggregation by anionic detergents of walls prepared according to their methods. Work (1961), on the other hand, found a complex spectrum of peptides to be released from walls of *Escherichia coli* by enzymes of lysozyme-like character, and interpreted these peptides as containing typical mucopolymer components linked to sets of amino acids derived from protein. Her conclusion was that 'each of the so-called "layers" contained mucopeptide components'.

Since our own results seemed to demonstrate clearly the existence of a separate mucopolymer layer in the walls of *Escherichia coli* (Weidel *et al.* 1960) and other Gram-negative micro-organisms (Martin & Frank, 1962*a*; Schocher, Bayley & Watson, 1962), the simplest interpretation of the findings of Shafa & Salton (1960) is that their wall preparations no longer contained an intact mucopolymer layer. As found recently in this laboratory, the empty Gram-negative cell wall does not necessarily disintegrate spontaneously even upon thorough destruction of its *R*-layer with lysozyme (Martin & Frank, 1962*b*). Hydrogen or other non-covalent bonding between components of the outer wall layers is sufficiently strong to keep them together, thereby preserving the shape of the cell wall fairly well; but by interfering with these weak bonds, mechanically or by anionic detergents, such a precarious structure disintegrates completely. It is easy to see how damage can be done inadvertently to the *R*-layer during wall preparation. For theoretical reasons, the cell must contain enzymes for the specific purpose of systematically hydrolysing certain covalent bonds which hold the *R*-layer together, otherwise the wall could not grow (Weidel *et al.* 1960). If not inactivated during wall preparation, these enzymes

can be expected to digest the *R*-layer, with the result that such cell walls can indeed be disintegrated by detergent alone. With Gram-negative organisms especially, the damaging effect of the enzymes is likely to be rapid and efficient, since the *R*-layer is extremely thin and closest to the cytoplasm, which is difficult to remove completely from the walls.

Two early observations were taken by us as directly indicating the presence and activity of such enzymes within *Escherichia coli*. First, most of the cell walls of *E. coli* in preparations made by our old method which involves a prolonged period of 'autolysis' under toluene (Weidel, 1951) are obtained as rounded-off rather than oblong forms. Secondly, cell walls prepared in this way yielded, on incubation of their phenol-insoluble fraction with lysozyme, proportions of the two main mucopolymer split products C_5 and C_6 (Primosigh, Pelzer, Maass & Weidel, 1961) which varied from preparation to preparation. In the hope of suppressing both effects, the procedure for wall preparation was changed so as to use the enzyme-inhibiting powers of sodium dodecylsulphate (SDS) at the earliest possible stage. When this was done, only oblong cell walls were obtained, in which the presence of a separate *R*-layer was now easily demonstratable.

Thus one is confronted with the apparent paradox that the very agent which in the hands of Shafa & Salton led to complete disintegration of their cell-wall preparations, served to preserve our walls in good shape. We thought it advisable therefore to investigate this paradoxical phenomenon further with one of Dr Salton's own cultures, and he kindly sent us his strain of *Salmonella gallinarum* for the purpose.

The R-*layer of* Salmonella gallinarum

The organisms were grown at 37° on a shaker for 9 hr. in nutrient broth (500 ml.), spun down and carefully resuspended in 200 ml. distilled water. To this suspension, 200 ml. of a 4% (w/v) SDS solution in water were added. The suspension became very viscous, and was allowed to stand overnight at about 20°, after which it was diluted to 1600 ml. with water and centrifuged at 66,000 ***g***. The sediment was taken up in 200 ml. water, the suspension treated briefly with DNAase and RNAase (Mg^{++} added) and centrifuged at 66,000 ***g***. The sediment was then resuspended in 4% (w/v) SDS solution, shaken for 1 hr. with glass beads in a Mickle disintegrator, washed three times with water on the centrifuge at 23,500 ***g*** and then shaken for 1 hr. in 4% (w/v) SDS solution. This was followed by six more washings on the centrifuge. Electron microscopic control showed the sediment now to consist of cell walls unchanged in shape, more than two-thirds of them completely empty (Pl. 1, fig. 1). Since we had the impression that most of the wall lipopolysaccharide had already been removed (contrary to the behaviour of *Escherichia coli* strain B lipopolysaccharide), phenol dissociation was omitted and the final proteolytic digestion effected immediately with either trypsin or pepsin. Either enzyme released material which still covered the mucopolymer layer of these walls without being arranged in a pattern of tightly packed spheres, as with *E. coli*. What was not solubilized of this material remained deposited on or beside the empty mucopolymer bags (Pl. 1, fig. 2), but could be removed now by washing the sedimented mixture three times at 23,500 ***g***, then shaking the deposit in 4% (w/v) SDS solution for 15 min. and washing again ten times at 66,000 ***g***. The final sediment consisted of pure mucopolymer layers of *Salmonella gallinarum* cell walls (Pl. 1, fig. 3).

As expected, this material dissolved completely in a buffered solution of lysozyme. The chromatographic pattern of ninhydrin-positive split products yielded by the lysozyme treatment had a marked qualitative and quantitative resemblance to that obtained with a lysozyme digest of the *Escherichia coli* mucopolymer layer (Primosigh *et al.* 1961; Pl. 2, fig. 4). There was only one major spot (X) between C_3 and C_4 which was either absent from or much weaker on the *E. coli* chromatogram. The similarity of the two patterns was confirmed by the determination of the molar ratios of muramic acid, glutamic acid, diaminopimelic acid and alanine in a hydrolysate of pure Salmonella mucopolymer (methods described by Primosigh *et al.* 1961). In this sequence, they were found to be 1·02:1·01:0·99:1·00:1·84 (mean of four determinations). Comparable protein-free mucopolymer bags from *E. coli* B gave ratios of 1:1:1:1:1·8 (Martin & Frank, 1962*b*). Other amino acids were not detectable on the chromatograms run for quantitative analysis.

Disappearance of the R-*layer from unstabilized cell walls*

After establishing the presence in the *Salmonella gallinarum* cell wall of a continuous separate mucopolymer layer, walls of this organism were prepared according to Shafa & Salton (1960), i.e. Mickle disintegrator treatment of organisms suspended in distilled water, thorough washing of the cell-wall fraction and treatment with trypsin. Plate 2, fig. 5, shows an electronmicrograph of this cell-wall preparation, which was not further washed in order not to lose anything. The walls are definitely deformed, as compared to walls shown in Pl. 1, fig. 1. The turbid suspension of these cell walls when mixed with one-tenth of its volume of 4% (w/v) SDS solution cleared almost immediately, and electron microscopy of the mixture then revealed no structures resembling cell walls or mucopolymer bags among the debris (Pl. 2, fig. 6), thus confirming the findings of Shafa & Salton (1960) and, at the same time, our interpretation of this result.

DISCUSSION

The lesson to be learnt from the foregoing is that 'a cell wall is not a cell wall is not a cell wall', i.e. is not nearly as static an object as might be thought. Just because in the complete Gram-negative cell wall (as opposed to the wall of Gram-positive organisms) the mucopolymer layer is only a minor (yet functionally highly important) component, it is an especially sensitive indicator of shifts in the interplay of the enzymes building and rebuilding it. This is illustrated by the fact that we found the structure of the *Escherichia coli* mucopolymer to change even during the short time it takes to spin down the organisms from the liquid culture, resuspend them and treat them with SDS or heat for stabilization. Lysozyme digests of mucopolymer from such organisms contain the components C_5 and C_6 (fig. 4; see also Primosigh *et al.* 1961) in a molar ratio of about 1:2. Shortening the time between harvesting the organisms and blocking the degradative enzymes noticeably decreases the ratio. But in order really to 'freeze' the mucopolymer structure in the form in which it exists in the fully active cells, one has to squirt the culture into boiling water immediately after stopping aeration. Then the mucopolymer yields almost no C_5, but correspondingly more C_6 instead. This is explained by the finding of one of our colleagues that *E. coli* cells contain an enzyme capable or removing C-terminal D-alanine from the peptide chain of the C_6 subunit of the mucopolymer, thereby

changing it into a C_5 subunit (Pelzer, 1962). For structural analysis of the Gram-negative cell wall it is essential to keep in mind, in addition to the necessity of working with stabilized wall material, that the whole structure is established through the intricate co-operation of covalent and non-covalent bonds. This means that upon destruction of the supporting mucopolymer layer, e.g. with lysozyme, a host of wall components will be freed, most of which are anything but lysozyme split products. These non-mucopolymer components may have contributed to the multitude of peptides encountered by Work (1961) in her lysozyme digests of *E. coli* cell walls. Judging from the chemical nature of split products obtained, it seems possible that her cell-wall preparations contained a variety of active hydrolytic enzymes, including proteolytic ones. We feel that it is unsafe to draw conclusions about cell wall structure unless the various wall layers are separated as far as possible, before analysing their components (Weidel & Primosigh, 1957; Martin & Frank, 1962*b*).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Cell walls of *Salmonella gallinarum* after treatment in a Mickle disintegrator with sodium dodecylsulphate. × 32,000.

Fig. 2. Same as fig. 1, after treatment with trypsin. × 32,000.

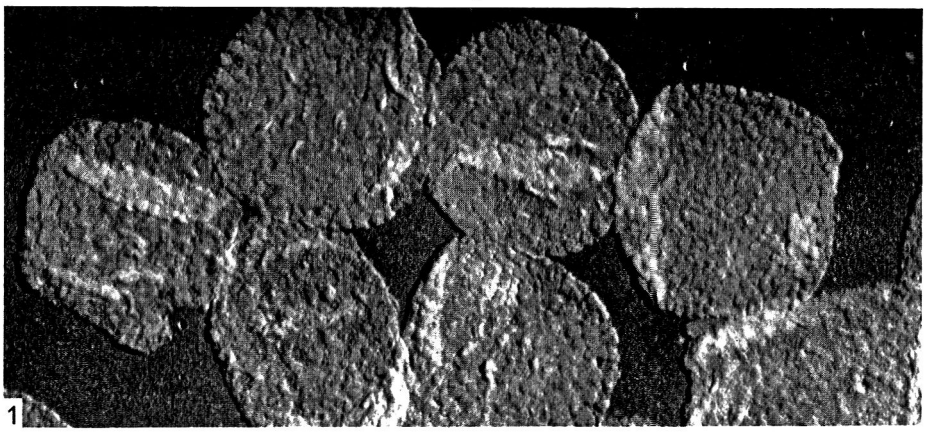
Fig. 3. Pure preparation of *Salmonella gallinarum* mucopolymer bags (*R*-layer). × 32,000.

PLATE 2

Fig. 4. Parallel chromatogram of lysozyme digests of *Escherichia coli* mucopolymer (C) and *Salmonella gallinarum* mucopolymer (S). Material in main spots of *Escherichia coli* chromatogram: *N*-acetyl-glucosaminyl-*N*-acetyl-muraminyl-L-alanyl-D-glutaminyl-*m*-diaminopimelic acid (upper spot = C_5); the lower spot (= C_6) contains the same mucopeptide with an additional D-alanine attached to DAP as C-terminal (cf. Primosigh *et al.* 1961; Pelzer, 1962).

Fig. 5. *Salmonella gallinarum* cell walls after Mickle disintegrator treatment and trypsin digestion. Sodium dodecyl sulphate was not used up to this stage. × 32,000.

Fig. 6. Same as fig. 5, after addition of sodium dodecyl sulphate. × 32,000.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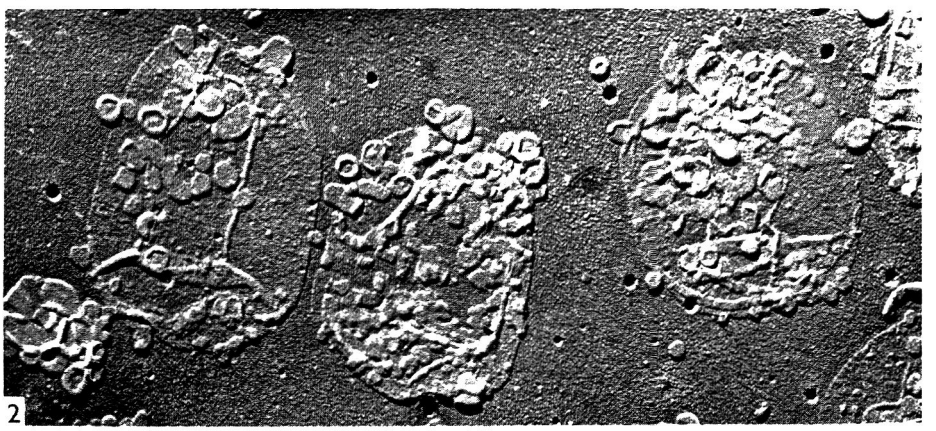
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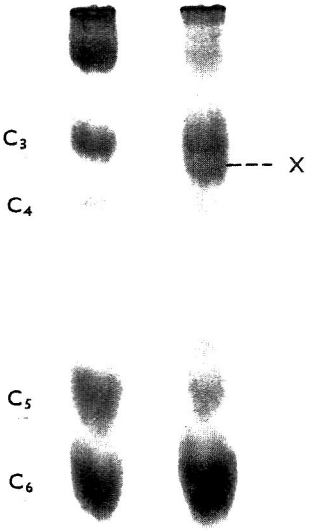
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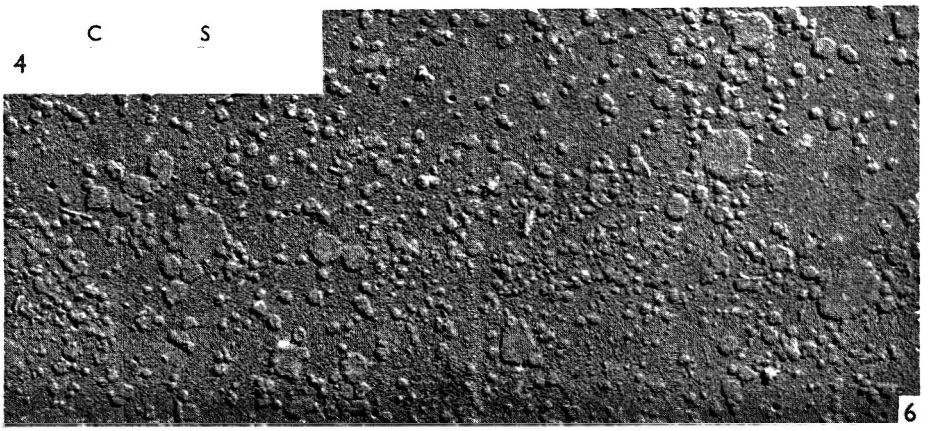
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Intracellular Sites of Synthesis of Encephalomyocarditis Virus Components in Krebs-2 Ascites Tumour Cells

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SUMMARY

Ascites tumour cells were homogenized and fractionated at various times after infection with encephalomyocarditis virus *in vitro*. Infective ribonucleic acid (RNA) could be obtained from the nuclear fraction soon after infection and the amount of infective RNA recovered from this fraction increased until a maximum was reached at about 4½ hr. From 4½ hr. onwards the amount of infective RNA associated with the nuclear fraction fell while that from the mitochondrial fraction increased, reaching a maximum at about 5½ hr. The amount of haemagglutinin and plaque-forming virus began to increase at about 4½ hr. and continued to rise until 8 hr. Most of this virus activity was found in the mitochondrial fraction, although from 6 hr. onwards an increasing amount was found in the microsomal fraction.

INTRODUCTION

Murine encephalomyocarditis (EMC) virus is a small ribonucleic acid (RNA) virus which can be grown under one-step conditions in agitated suspensions of ascites carcinoma cells (Sanders, Huppert & Hoskins, 1958). After infection there is an eclipse phase lasting 4–4½ hr. followed by intracellular accumulation of virus until a maximum is reached about 8 hr. after infection. From 8 to 12 hr. virus is released into the medium; at the same time the cells die.

Huppert & Sanders (1958) obtained infective RNA preparations by cold (4°) phenol extraction of infected cells, but similar preparations from partially purified EMC virus were not infective. This led the authors to postulate that the source of infective RNA in infected cells was not the virus, but an RNA component which might be a virus precursor. Bellett *et al.* (1962) have shown that infective RNA can be obtained from virus by using the improved cold phenol extraction described by Sanders (1960), and that the result obtained by Huppert & Sanders (1958) may have been due to ribonuclease in their preparations. The synthesis and subsequent fate of virus precursor RNA have, therefore, been followed by studying changes in the amount of infective RNA which occur without corresponding changes in virus titre. Such studies of ascites cells infected by EMC virus confirm that the synthesis of infective RNA begins 1–2 hr. before virus formation begins, and is complete 3 hr. before virus reaches its maximum titre (Sanders, 1960). Martin & Work (1962) showed that 60% of the RNA destined to be incorporated into virus particles has been synthesized before virus protein synthesis begins 4 hr. after infection. Cells inhibited by the addition of euflavine 4–5 hr. after infection produce the normal amount of infective RNA, but no haemagglutinin or infective virus (Sanders, 1960).

As a working hypothesis, the multiplication of EMC virus in this system has therefore been divided into the following stages given in chronological order:

(1) synthesis of a precursor, consisting of or containing infective RNA,

(2) synthesis of haemagglutinin,

(3) assembly of RNA and protein into plaque-forming virus particles.

Experiments reported here were designed to determine in which part of the infected cell each stage occurs.

METHODS

Cell cultures and virus growth. Krebs-2 ascites cells at a concentration of 10^8/ml. in phosphate buffered saline (PBS) were infected with 3 plaque forming units (pfu) of EMC virus (K-2 strain)/cell and left at 4° for one or more hours. Storage at 4° for up to 16 hr. had no effect on subsequent virus yield. These infected cells were then diluted rapidly to 10^7/ml. in Earle's saline at 37° and maintained in suspensions; one-step growth of virus with maximum possible synchrony of multiplication occurred under these conditions. Thus, times after infection referred to were times after transfer to 37°. After virus growth the cultures were chilled rapidly and kept at 0° during all subsequent procedures. Cells were harvested, washed once in PBS free of calcium and magnesium and resuspended to a concentration of 10^8/ml. A sample was removed as a control; the remainder was lysed and separated into sub-cellular fractions.

Lysis and fractionation of cells. Cells were lysed by double osmotic shock followed by homogenization (Martin, Malec, Coote & Work, 1961). The lysate was centrifuged at 500*g* (max.) for 5 min. at 0°, the deposited nuclear fraction washed once in about 5 vol. of a solution containing 0·25 M-sucrose and 0·1 M-KCl, and the washings added to the supernatant fluid. The crude nuclei were further purified by homogenization at *c.* 1000 rev./min. for 15 sec. in 10 vol. of 2 M-sucrose, followed by centrifugation at 40,000*g* for 30 min. Nuclei were deposited while whole cells and nuclei with attached cytoplasm formed a band at the top of the tube (Busch, Starbuck & Davis, 1959). The purified nuclei were washed in the sucrose KCl solution and resuspended in this solution to give about 5×10^7 nuclei/ml.

The cell homogenate after removal of nuclei at 500*g* was centrifuged at 9,000*g* for 15 min. then at 144,000*g* for 45 min. to deposit the mitochondrial and microsomal fractions, respectively, leaving the supernatant fluid as cell sap. The mitochondrial and microsomal fractions were resuspended in the same volume of 0·25 M-sucrose + 0·1 M-KCl as that used for the nuclei. Samples were removed for deoxyribonucleic acid (DNA) and succinic dehydrogenase determinations, and the remainder was stored at −20° until used for RNA extraction and haemagglutinin and plaque titrations.

Purity of the fractions. We have followed the common practice of calling the 500*g*, 9000*g* and 144,000*g* deposits the nuclear, mitochondrial and microsomal fractions respectively, to indicate the main constituents of the fractions. The material not sedimented at 144,000*g* we have called cell sap. Each of these fractions probably contains minor components, and the main constituent of a fraction is not necessarily involved in synthesis of virus material.

Since our results were affected by contamination of one fraction by another, it was necessary to estimate the extent of such contamination. We attempted to do this by cytological and biochemical investigations of the fractions. Haemocytometer counts

of the crude lysates after staining for 5 min. in 10 vol. of 0·2% (w/v) nigrosin in 0·3M-KCl showed that they usually contained more than 95% nuclei and less than 5% whole cells. Similar counts of the nuclear fraction after purification showed on average 86% nuclei without cytoplasmic attachments, 9% nuclei with cytoplasmic attachments, 5% free cytoplasmic fragments and less than 1% whole cells based on counts of about 100 nuclei. These results were confirmed by observing unstained material with the phase-contrast microscope. About 0·7% of the nuclei in the crude lysate was deposited with the mitochondrial fraction as shown by staining with two volumes of 0·2% (w/v) nigrosin.

The contamination of other fractions by mitochondria can be estimated by their content of succinic dehydrogenase, a known mitochondrial enzyme, determined by the method of Aldridge & Johnson (1959). The nuclear fraction usually contained about 10% and microsomes and cell sap up to 2·0% of mitochondria or unbroken cells. Contamination in excess of these figures is mentioned in the text. Similarly, DNA was used as a measure of nuclear contamination of the cytoplasmic fractions. DNA was extracted by the Schneider technique as modified by Ogur & Rosen (1950) and determined by the method of Burton (1956). Over 96% of the DNA was found in the nuclear fraction, between 0·3–2·8% in the mitochondrial fraction, 0·4–1·4% in the microsomal fraction, and 0·2–1·1% in the cell sap. Nuclear contamination of cytoplasmic fractions was, therefore, not significant.

Infectious RNA. This was prepared by phenol extraction at 4° (Gierer & Schramm, 1956; Sanders, 1960) with 0·25M-sucrose + 0·1M-KCl as the suspending medium during extraction. The concentration of RNA was estimated by optical density measurements at 260 mμ. All samples had spectra typical of RNA. Infectivity of RNA preparations was estimated by mixing dilutions in PBS with an equal volume of Krebs-2 ascites cells (10^8/ml.) in PBS. Five mice per dilution were inoculated intraperitoneally with 0·2 ml. of mixture after it had stood at room temperature for 30 min. (Huppert & Sanders, 1958). LD50 endpoints were calculated by the method of Reed & Muench (1938). Results were expressed in terms of infectivity (LD50/10^8 cell equivalents), and specific infectivity (LD50/μg. RNA) which allows for differences in recovery of RNA. A fraction in which infective RNA is concentrated can be identified since its specific infectivity will exceed that of whole cells. Specific infectivity may, however, obscure the importance of a fraction rich in non-infective RNA (e.g. microsomes) so that it is also necessary to consider results in terms of infectivity per 10^8 cell equivalents.

Haemagglutinin tests were performed in MRC pattern plastic trays with sheep red blood cells (0·1%, v/v). The diluent was one part glucose solution (4·5%, w/v) + 1 part PBS with 0·05% (w/v) gelatin added.

Virus titrations were by a plaque technique using Krebs-2 ascites cells in agar suspension (Sanders *et al.* 1958). Plates were incubated in a sealed vessel containing CO_2 buffer (Bellett, 1960).

RESULTS

Absence of non-specific adsorption by cell fractions. One possible disadvantage of cell fractionation in the study of the intracellular location of virus materials is non-specific adsorption of these materials by cell particles after homogenization of the cells (Stickl, quoted by Breitenfeld & Schäfer, 1957). Two experimental facts suggest

that non-specific adsorption is not significant in the EMC-ascites cell system. First, the distribution of virus materials between cell fractions varies with time after infection. Secondly, virus added directly to a cell homogenate sedimented with the microsomes as expected and showed no evidence of non-specific adsorption (Table 1).

Infective RNA in the nuclear fraction immediately after infection. When cells were fractionated after 30 min. contact with virus at 4°, infective RNA was recovered from the nuclear fraction while RNA from other fractions was non-infective (Table 2). The specific infectivity (LD 50/μg.) of nuclear RNA was greater than that of whole cell RNA since the latter was diluted with non-infective RNA from other fractions. After a further hr. at 4°, the infectivity of RNA from the nuclear fraction did not increase, but a small amount of infectivity was detected in the mitochondrial fraction, not all of which could be accounted for by nuclear contamination. The specific infectivity of RNA from the mitochondrial fraction did not exceed that from whole cells.

Table 1. *The distribution of virus added to an homogenate of uninfected cells*

	Virus	
Cell fraction	Haemagglutinating units total	Plaque forming units total ($\times 10^{-5}$)
Nuclei	25	20
Mitochondria	200	200
Microsomes	800	1950
Sap	< 175	250

Virus (2000 haemagglutinating units, 3×10^8 plaque forming units) was added to an homogenate of Krebs-2 ascites cells which was then centrifuged to give subcellular fractions.

Table 2. *Infective RNA in the nuclei of cells infected at 4°*

	30 min.		90 min.	
Cell fraction	Infectivity	Specific infectivity	Infectivity	Specific infectivity
Whole cells	32	0·026	68·0	0·072
Nuclei	69	0·220	24·6	0·160
Mitochondria	0	0	1·0	0·015
Microsomes	0	0	0	0
Cell sap	0	0	0	0

Cells were infected with 3 p.f.u. of virus per cell and kept at 4° for the stated times. The cells were then lysed and fractionated. RNA was extracted from the fractions, its concentration was estimated by optical density measurements at 260 mμ and its infectivity for mice was determined. Infectivity, LD 50/10^8 cell equivalents; specific infectivity, LD 50/μg. RNA.

Synthesis of infective RNA in the nuclear fraction of infected cells during the eclipse phase. Synthesis of infective RNA apparently occurred in the nuclear fraction when infected cells were transferred from 4° to 37°. The infectivity of RNA from the nuclear fraction increased rapidly from about 3 hr. after transfer to 37° (Fig. 1), reaching a maximum at about 4½ hr. and exceeded the specific infectivity of whole cell RNA during this period (Fig. 2). The infectivity of RNA from the mitochondrial fraction increased slightly during the eclipse phase but did not exceed the specific infectivity of whole cell RNA. Microsomal and cell sap RNA showed little infectivity throughout the eclipse phase.

Association of infective RNA with the mitochondrial fraction after the eclipse phase. There was a loss of infective RNA from the nuclear fraction between $4\frac{1}{2}$ and 8 hr. after infection (Figs. 1 and 2). This was accompanied by an increase in both the infectivity and specific infectivity of RNA extracted from the mitochondrial fraction from $4\frac{1}{2}$ to $5\frac{1}{2}$ hr. No comparable increase occurred in the infectivity of microsomal or cell sap RNA. The amount of infective RNA recovered from all fractions

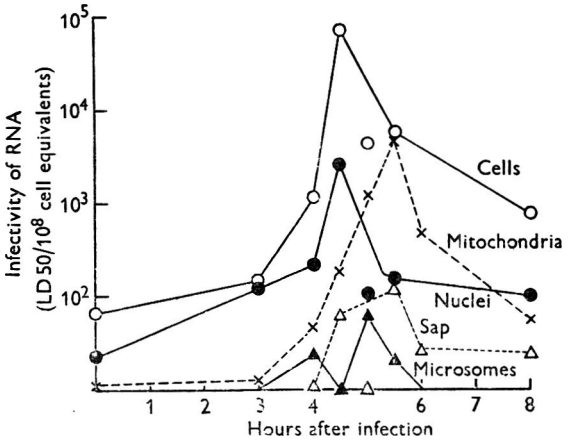

Fig. 1. Infectivity (LD 50/10^8 cell equivalents) of RNA extracted from fractions of infected cells during virus growth. Hours after infection refers to time of incubation at 37°. ○—○, cells; ●—●, nuclei; ×- - -×, mitochondria; ▲—▲, microsomes; △- - - -△, sap.

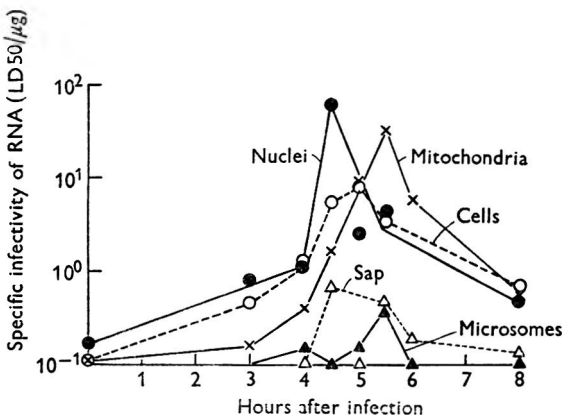

Fig. 2. Specific infectivity (LD 50/μg.) of RNA extracted from infected cells during virus growth. ○- - -○, cells; ●—●, nuclei; ×—×, mitochondria; ▲—▲, microsomes; △- - - -△, sap.

decreased from $5\frac{1}{2}$ to 8 hr. This loss was also reported by Sanders (1960) who suggested it was due to incorporation of RNA into virus particles, whence it could not be extracted in an infective form with cold phenol. While we can now obtain infective RNA from virus by the technique used in this paper, it still seems that it may be more difficult to obtain it from virus within cells $5\frac{1}{2}$ to 8 hr. after infection

than from precursor RNA or extracellular virus. From 10^9 pfu of extracellular virus we usually obtained about 10^5 LD50 of infective RNA. However, from 10^9 pfu of virus within 8 hr. infected cells we obtained only 3×10^3 LD50 of RNA. The reduced recovery of RNA from all fractions after $5\frac{1}{2}$ hr. may, therefore, be due to assembly of the RNA component into virus particles which, within cells, are apparently more resistant to phenol extraction.

Association of haemagglutinin with mitochondria after the eclipse phase. Haemagglutinin increased rapidly from about $4\frac{1}{2}$–8 hr.; most of the haemagglutinin was found in

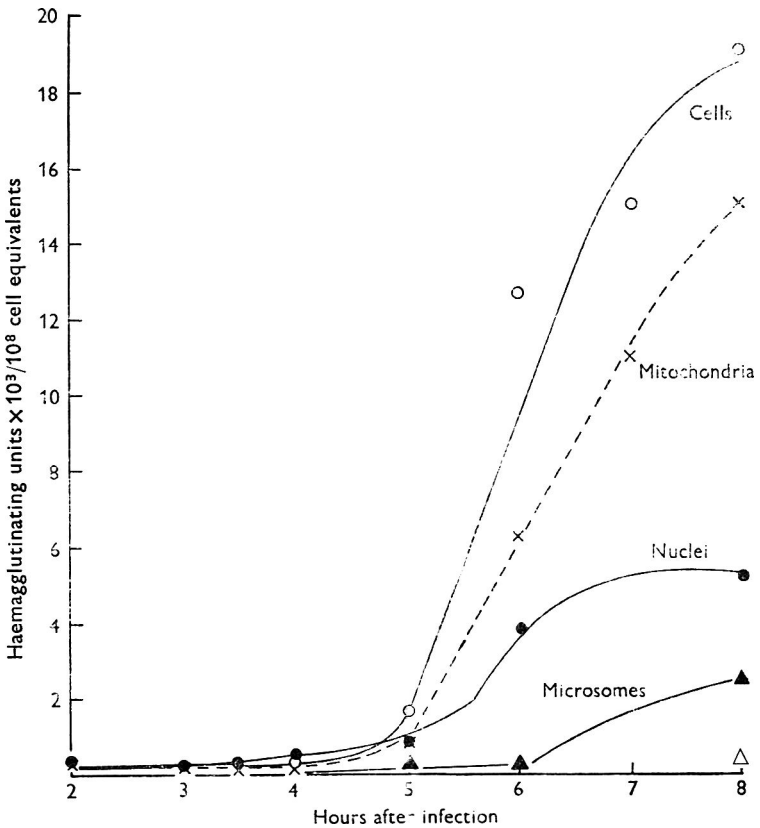

Fig. 3. Haemagglutinin in fractions of infected cells during virus growth. ○—○, cells: ●—●, nuclei; ×—×, mitochondria; ▲- - -▲, microsomes; △, sap.

the mitochondrial fraction, which showed the greatest increase in titre during this period (Fig. 3). The nuclear fraction also contained haemagglutinin which increased in titre during the same period but neither the amount of haemagglutinin nor its increase was as great as that found in the mitochondrial fraction. Further, some of this haemagglutinin may have been due to cytoplasmic contamination, since 19% of the succinic dehydrogenase activity was found in the nuclear fraction at 8 hr. The microsomal fraction showed an increase in haemagglutinin from 6 to 8 hr. after infection, although the total amount was still low; this increase was probably not due to cytoplasmic contamination ($< 1\%$). This suggests that some haemagglutinin

had been incorporated into virus particles which were then liberated from the mitochondrial fraction and so were deposited with the microsomes which have similar sedimentation properties (Table 1).

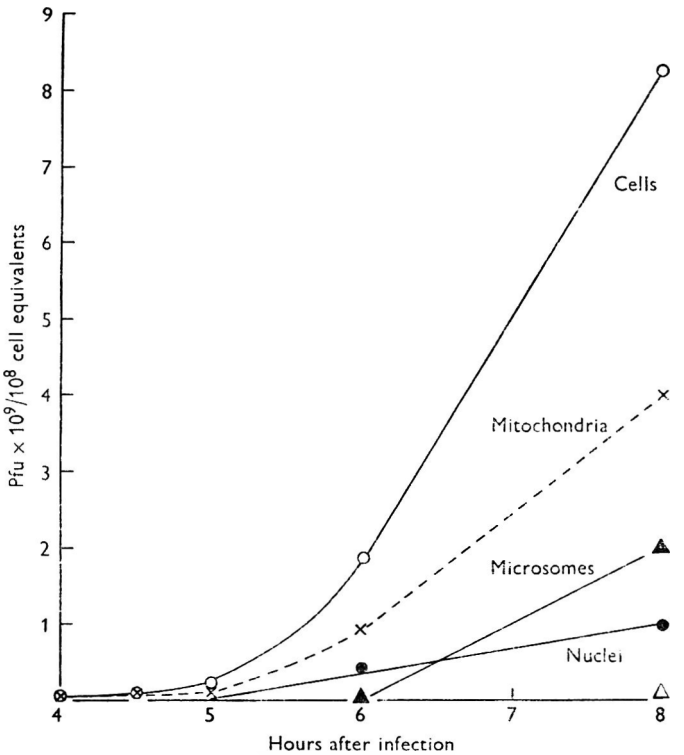

Fig. 4. Plaque-forming virus in fractions of infected cells during virus growth. ○—○, cells; ●—●, nuclei; ×– – –×, mitochondria; ▲—▲, microsomes; △, sap.

Association of virus progeny with the mitochondrial fraction. Infective (plaque-forming) virus was detected in the mitochondrial fraction at 4½ hr. after infection (Fig. 4) and increased rapidly up to 8 hr. after infection, representing a substantial proportion of the virus found in the whole cell control. The nuclear fraction also contained virus; this showed little increase in titre and, as suggested in the case of haemagglutinin, may have been due to cytoplasmic contamination at 8 hr. Little virus was detectable in the microsomal fraction before 6 hr. but, between 6 and 8 hr. after infection a marked increase in titre occurred, reaching 30 % of the total virus at 8 hr. This increase was greater than the increase in haemagglutinin of the microsomal fraction and provided further evidence that completed virus particles were released from the mitochondrial fraction and were then deposited with the microsomes on centrifugation (Table 1).

DISCUSSION

The infective RNA obtained from the nuclear fraction after infection of cells at 4° may be derived from complete virus particles or from some component (which may be RNA) released by them. However, the RNA synthesized during the first 4½ hr.

after transfer to 37° appeared to be in the form of RNA component and not virus, since increase in the amount of infective RNA obtained was not accompanied by a corresponding increase in haemagglutinin or plaque-forming virus. Increase in the infectivity of nuclear RNA between 0 and 4½ hr. and the subsequent decrease may then be interpreted as synthesis of the RNA component in the nuclei and the transfer of this component to the cytoplasm to initiate the production of haemagglutinin and infective virus. Another interpretation is that the RNA is synthesized elsewhere and concentrated in the nuclear fraction; but this is unlikely since other fractions are virtually free of infective RNA until 4 hr. after infection. It has also been suggested (Franklin & Rosner, 1962) that our results might have been due to contamination of our nuclear fraction by viral RNA of cytoplasmic origin. We attempted to assess this type of contamination in the nuclear fraction by measuring the activity of succinic dehydrogenase, which is a mitochondrial enzyme. The amount of infective RNA recovered from the nuclear fraction could not be correlated with the amount of cytoplasmic contamination in a series of experiments at a given time after infection. Further, in one experiment, although there was no succinic dehydrogenase activity in the nuclear fraction, three times more infective RNA was recovered from this fraction than from all the cytoplasmic fractions put together. In another experiment infective RNA was recovered immediately after infection only from the nuclear fraction, whereas if this RNA resulted from contamination with cytoplasmic material, some infective RNA should also have been obtained from the cytoplasmic fractions.

Some of the infective RNA obtained from the mitochondrial fraction from 5 hr. onward may come from virus although the bulk of the RNA must be from the RNA component up to 5 hr. since little virus is detectable during this time. It thus appears that the RNA component is transferred from the nuclear fraction to the mitochondrial fraction and only then does synthesis of haemagglutinin and production of complete virus begin.

The synthesis of haemagglutinin and assembly of virus components into plaque-forming particles took place in the cytoplasm and was associated with the mitochondrial fraction. This result was also obtained by Martin & Work (1961), and can be interpreted in at least four ways:

(1) Virus material free in the cell was non-specifically adsorbed by the mitochondria which are not involved in virus synthesis. The results of an experiment in which virus was added to a homogenate of uninfected cells (Table 1) suggest that this is untenable.

(2) Virus was produced in packets which sedimented with the mitochondria.

(3) Some virus-synthesizing organelle containing virus material sedimented with the mitochondria.

(4) Mitochondria, themselves, were the sites of synthesis of haemagglutinin and of assembly of RNA component and haemagglutinin into the mature virus particles.

It will be impossible to distinguish between the last three interpretations until techniques are available which yield guaranteed pure fractions and yet are sufficiently rapid to preserve virus activity. In the absence of such techniques, we must emphasize that the term mitochondrial fraction includes all cytoplasmic particles sedimented at 9000*g*. No evidence was obtained suggesting that the microsomal (144,000*g*) fraction was in any way involved in the synthesis of EMC virus although

virus was deposited with the microsomes from cells fractionated between 6 and 8 hr. after infection (Fig. 4). Microsomes and EMC virus have similar sedimentation properties; 70–80 % of the virus was recovered from the microsomal fraction when virus was added to an uninfected cell homogenate which was then fractionated (Table 1). Any virus free in the cell would be expected to deposit with the microsomes and therefore that found in the microsomal fraction obtained from 6 to 8 hr. infected cells was possibly mature virus recently liberated from the mitochondrial (9000*g*) particles.

Sanders (1960) suggested that haemagglutinin synthesis and virus assembly are separate processes in EMC virus multiplication. Additional evidence for this view is that the ratio of haemagglutinin to plaque-forming titre was much higher in our mitochondrial fraction than in the microsomal fraction 8 hr. after infection. This suggests that the mitochondrial fraction contained haemagglutinin not yet incorporated into plaque-forming virus particles, although no difference was detected between the times of appearance of haemagglutinin and virus.

Biochemical changes induced in Krebs ascites tumour cells on infecting with EMC virus have been studied using ^{14}C-labelled orotic acid and valine (Martin & Work, 1961). Changes in the rate of incorporation of labels into cell materials after infection obscured incorporation into virus components and the movement of these components within the cell. However, the reported loss of phosphate soluble, nuclear RNA which was compensated by a similar rise in mitochondrial RNA possibly represented the transfer of infective RNA component from the nucleus to the mitochondrial fraction that we observed. Increased incorporation of orotic acid into cytoplasmic RNA during the appearance of virus was not due to viral RNA synthesis since this had already taken place (Martin & Work, 1962).

Our results using biological markers suggest a hypothesis for the multiplication of EMC virus which has as its main features the replication of the RNA component in the nucleus of the cell during the eclipse phase and the transfer of this component to the cytoplasm where it may initiate and act as a template for the synthesis of haemagglutinin. The RNA component and haemagglutinin are then incorporated into infective particles. Similar conclusions have been reached by Martin & Work (1961, 1962).

Franklin & Rosner (1962) have studied by autoradiography the incorporation of (^{3}H)uridine into L cells infected by Mengo virus, which is closely related to EMC. They found decreased nuclear incorporation, followed by stimulated cytoplasmic incorporation during and after the appearance of virus, thus confirming the results obtained by Martin & Work (1961). Franklin & Rosner, unlike Martin & Work, concluded that synthesis of virus RNA was cytoplasmic.

It has been amply demonstrated that RNA synthesis precedes the appearance of EMC virus in infected ascites cells (Sanders, 1960; Martin & Work, 1962; this paper). Since in the L cells-mengo virus system virus was detected 2 hr. after infection, it may be presumed that only changes occurring in the first two hours have any relevance to viral RNA synthesis. During this time the stimulation of cytoplasmic incorporation as measured by autoradiographic techniques amounted to less than 2 photographic grains per cell (Franklin & Rosner, 1962), whereas after virus multiplication was complete a stimulation of 50–60 grains per cell was observed. It seems that the experiment of Franklin & Rosner has detected a gross disturbance of the

nucleic acid metabolism of the host cells, but gives no indication of the site of viral RNA synthesis.

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Nutritional Requirements and Metabolism of *Mycoplasma laidlawii*

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SUMMARY

A study of the nutritional requirements of *Mycoplasma laidlawii* has led to the development of a growth medium containing inorganic salts, amino acids, glucose, nucleosides, vitamins and 1% (w/v) bovine serum albumin fraction V. No cholesterol was detected in this medium. Potassium, magnesium and phosphate ions were essential growth requirements. Sodium chloride provided the high tonicity required by the growth medium. The addition of an ammonium salt to the medium enabled the growth of the test organism in the presence of 13 amino acids. Resting organisms of *M. laidlawii* did not catabolize any of the 22 different amino acids tested under conditions which allowed amino acid degradation by *Mycoplasma hominis*. Glucose was essential for growth and could be replaced by maltose but not galactose, fructose, mannose, lactose or sucrose. The requirement for nucleic acid precursors was satisfied by adenosine, guanosine and cytidine, and that for vitamins by nicotinic acid, riboflavin, folinic acid, pyridoxine, pyridoxal and thiamine. Cholesterol was not necessary for growth of the test organism.

INTRODUCTION

The nutritional requirements of the Mycoplasmataceae are of special interest since they are the smallest autonomous self-replicating organisms known. Our knowledge concerning the nutritional requirements of this group is still fragmentary and based on experiments performed with partially defined media (Adler & Shifrine, 1960; Razin, 1962*a*). An attempt to compound a chemically defined culture medium for Mycoplasma was made by Razin & Knight (1960*a*) with the saprophytic *Mycoplasma laidlawii* as the main test organism. The medium obtained was only partially defined, but enabled the determination of nucleic acid precursor requirements of the saprophytic Mycoplasma (Razin & Knight, 1960*b*; Razin, 1962*b*). The aim of the present work was to render the partially defined medium of Razin & Knight (1960*a*) as chemically defined as possible. Various modifications of this medium led to the development of a 'minimal' medium, which enabled the determination of inorganic ion, carbohydrate, amino acid, nucleic acid precursor and vitamin requirements of *M. laidlawii*. In order to relate the observed nutritional requirements to metabolic activity, the amino acid metabolism of *M. laidlawii* was also investigated.

The 'minimal' medium obtained proved to be cholesterol-free and was thus suitable for ascertaining the cholesterol requirement. The problem of cholesterol

requirement by saprophytic Mycoplasma is of great theoretical interest. The parasitic Mycoplasma have been shown to require cholesterol or related sterols for growth (Edward & Fitzgerald, 1951; Smith & Lynn, 1958; Rodwell, 1956). As hitherto no other group of bacteria is known to require cholesterol, it was suggested to use this requirement as a criterion for distinguishing *Mycoplasma* spp. from bacteria and L-forms (Edward & Freundt, 1956). The possibility that the saprophytic Mycoplasma do not require cholesterol was already indicated by several authors (Razin & Knight, 1960*a*; Rothblat & Smith, 1961). Growth of *M. laidlawii* in the 'minimal' medium has proved that this saprophytic strain does not require cholesterol for growth.

METHODS

Organisms. *Mycoplasma laidlawii* strain A (PG 8) was kindly provided by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent); in the sequel '*Mycoplasma laidlawii*' will refer to this strain. *Mycoplasma hominis* strain no. 23 was isolated in our laboratory from a case of vaginitis.

Media. A modified Edward medium (Razin & Oliver, 1961) was used for keeping stock cultures and growing the organism used in the nutrition and metabolic experiments. The same medium, solidified with 1·25% (w/v) Bacto-agar (Difco, certified) was used for viable counts (Butler & Knight, 1960). The partially defined medium described by Razin & Knight (1960*a*) and Razin (1962*b*) served as a basis for the nutritional studies. This medium will be referred to as the partially defined medium.

Pyrex glassware was used in all experiments. Before use it was cleaned in a chromic + sulphuric acid mixture and thoroughly rinsed in distilled water. The tested medium was dispensed in 10 ml. quantities into $6 \times \frac{5}{8}$ in. sterile screw-capped test tubes. Sterilization of heat-labile substances was carried out by suction through sintered glass filters or by steaming for 20 min. (Merrifield & Dunn, 1950).

Conditions of growth. The organisms were grown in 5 ml. liquid Edward medium for 24 hr. at 37°, harvested and washed according to Butler & Knight (1960). Each tube of experimental medium received 0·1 ml. of a 1/10 dilution of the washed organisms. The initial inoculum contained about 10^5 viable particles/ml. medium. Inoculated test tubes were incubated statically in air at 37°. Growth was usually estimated after incubation for 96 hr.

Assessment of growth. Growth in experimental media was measured by titration of the acid formed by the organisms during growth (Razin & Knight, 1960*a*) or by the viable count technique as described by Butler & Knight (1960).

Suspensions of organisms. The organisms were grown in 1–2 l. quantities of liquid Edward medium in gently rotated Erlenmeyer flasks. *Mycoplasma laidlawii* was harvested after 24 hr. and the slower growing *M. hominis* after 48 hr. of incubation at 37°. The organisms were sedimented by centrifugation at 9000 *g* for 10 min. at 6° and resuspended in 0·067 M-phosphate buffer (pH 7·8). Since washing with buffer decreased the metabolic activity of the organisms very markedly, they were used unwashed. Suspensions of the organism in buffer were made to contain 1 mg. N/ml. Cellular-N was determined by comparing turbidity at 420 mμ to a previously prepared standard curve which related turbidity and cellular-N as determined by the micro-Kjeldahl procedure (Ma & Zuazaga, 1942).

Cell-free extract. A very thick suspension of *Mycoplasma laidlawii* was crushed

without abrasives in a Hughes press previously cooled to $-35°$ (Hughes, 1951). The intact organisms and debris were sedimented by centrifugation at 10,000 *g* for 20 min. The resulting supernatant fluid was opalescent.

Chemicals. Most of the chemicals used were analytical reagents. Crystallized bovine plasma albumin was the product of Armour Pharmaceutical Co. (Kankakee, Illinois, U.S.A.). Albumin (bovine) powder fraction V was obtained from the Mann Research Laboratories Inc. (New York 6, N.Y., U.S.A.). Defatted albumin fraction V was prepared by extraction of the dry powder with methanol and ether (Gilby, Few & McQuillen, 1958). Dialysis of human serum and albumin fraction V was carried out as described previously (Razin & Knight, 1960*a*). Dialysis was improved by constant stirring of the dialysing medium. The dialysed serum and albumin solutions were sterilized by filtration through sintered glass filters. The dialysed serum was kept at $-10°$ until used.

Manometric methods. Oxygen uptake was measured by the conventional Warburg manometric technique (Umbreit, Burris & Stauffer, 1957). Each Warburg vessel contained 10 μmole substrate, 1 ml. cell suspension (equiv. 1 mg. N), 1 ml. of 0·067 M-phosphate buffer (pH 7·8) and 0·1 ml. of 1×10^{-4} M-pyridoxal phosphate; the total volume was 3·2 ml.; 0·2 ml. of 15% (w/v) KOH was in the centre well. Incubation was carried out in air at 30° for 3 hr. All manometric data were corrected for the endogenous rates.

Transamination. This was tested in Thunberg tubes with hydrogen as the gas phase. Each tube contained 0·5 ml. of cell-free extract, 0·2 ml. of 0·067 M-phosphate buffer (pH 7·8), 10 μmole sodium pyruvate, 10 μmole glutamic acid or aspartic acid and 0·01 μmole pyridoxal phosphate; total volume 1·2 ml. Incubation was carried out at 37° for 3 hr.

Determination of amino acids. Paper chromatography was used for qualitative and quantitative determinations of amino acids and their degradation products (Giri, Radhakrishnan & Vaidyanathan, 1952). The standard solvent was butanol + acetic acid + water (40 + 10 + 50, by vol.). The papers were sprayed with 0·5% (w/v) ninhydrin in acetone. Proline was identified by spraying the paper with 0·2% (w/v) isatin in acetone (Block, Durrum & Zweig, 1958). For the identification of citrulline the paper was sprayed with 1% (w/v) *p*-dimethylaminebenzaldehyde in N-HCl (Block *et al.* 1958).

Ammonia production was estimated by nesslerization (Umbreit, Burris & Stauffer, 1957).

Total cholesterol. Fifty ml. of the minimal medium (Table 2) were freeze-dried. The lipid of the resulting solid residue was extracted by methanol and ether (Gilby *et al.* 1958). The etheral extract was saponified to liberate esterified cholesterol and the total cholesterol determined by digitonin precipitation and the Liebermann–Burchard test, as outlined by Cook (1958).

RESULTS

Inorganic ion requirements

The phosphate buffer included in the partially defined medium of Razin & Knight (1960*a*, Table 1) has a low buffering capacity at pH values near 8·0, which is the optimal pH for growth of *Mycoplasma laidlawii* (Razin & Oliver, 1961) and becomes

growth-inhibitory at concentrations higher than 0·04M (Razin & Knight, 1960*a*). 2-Amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, with optimal buffering capacity at pH 8·0, was therefore tried as a substitute for phosphate buffer. Figure 1 shows that growth of *M. laidlawii* was markedly improved both in the rate and in the total growth when phosphate buffer was replaced by tris buffer. The optimal concentration of tris buffer for growth was found to be 0·05M. Tris buffer was used instead of phosphate buffer in all subsequent experiments.

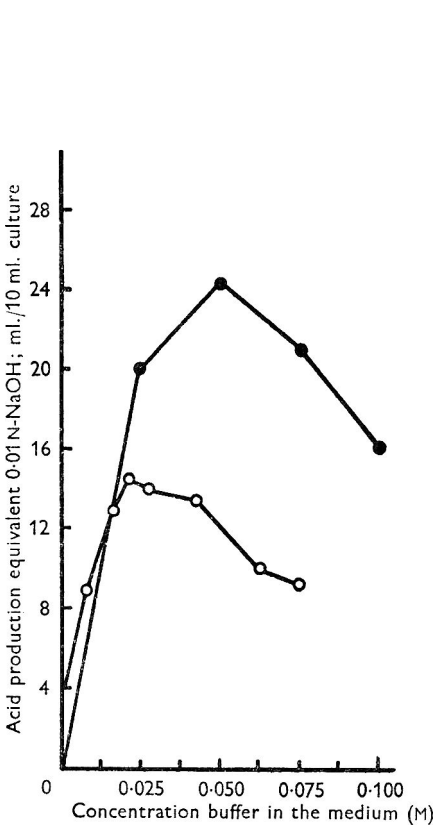

Fig. 1

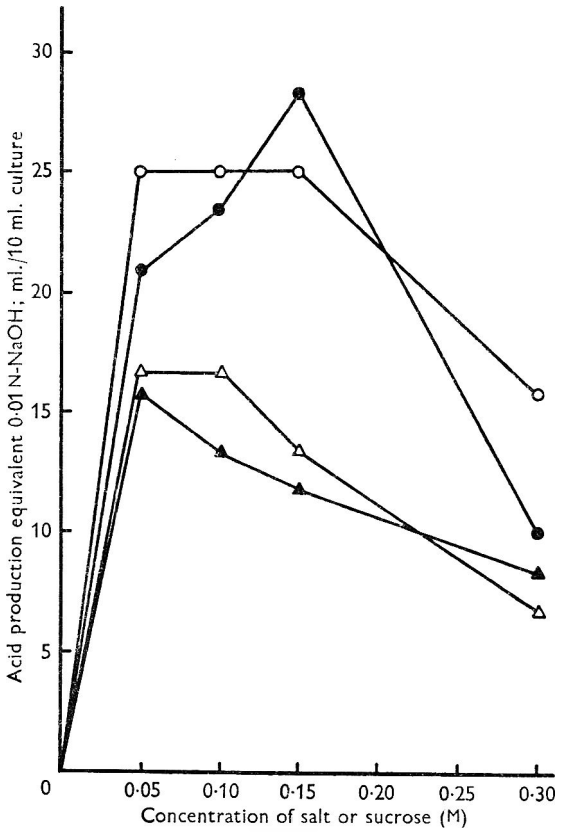

Fig. 2

Fig. 1. Growth of *Mycoplasma laidlawii* in partially defined medium containing 10 % (v/v) human serum and different amounts of phosphate buffer (○) or tris buffer (●) at pH 8·3.

Fig. 2. Effects of: sodium chloride (●); sodium acetate (○); sodium lactate (△); sucrose (▲); on the growth of *Mycoplasma laidlawii* in partially defined medium from which sodium chloride was omitted. The medium contained 10 % (v/v) human serum and salt-free Bacto-vitamin free Casamino acids.

The role of sodium chloride included in the partially defined medium was tested by its omission from the growth medium. For this experiment the Bacto-vitamin free Casamino acids which contain about 38% (w/w) NaCl, was replaced by salt-free Bacto-vitamin free Casamino acids. As seen from Fig. 2, no growth of the test organism took place when NaCl was omitted. Optimal growth was obtained

with 0·15 M-NaCl. Sodium acetate, and to a less extent sodium lactate and sucrose, may replace NaCl in the growth medium (Fig. 2). Spermine, which is known to protect fragile organisms and organelles from lysis in hypotonic media (Tabor, Tabor & Rosenthal, 1961) could not replace NaCl in the medium. Spermine became growth-inhibitory at concentrations of 10 μg./ml. or higher.

No growth of the test organism occurred when KCl was omitted from the growth medium (Fig. 3). *Mycoplasma laidlawii* did not grow when magnesium sulphate or

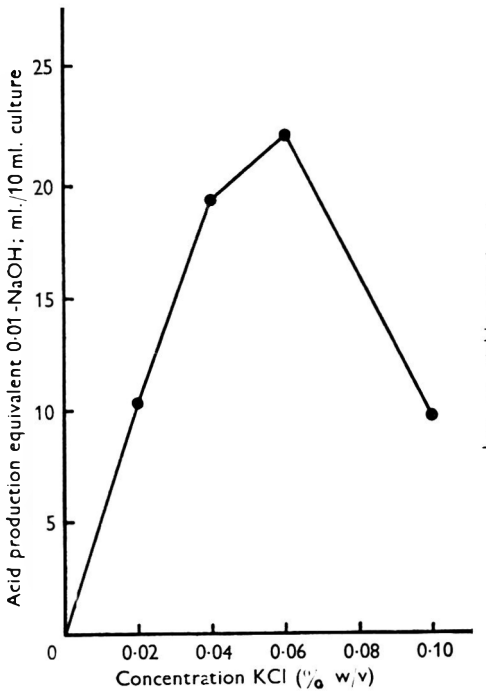

Fig. 3

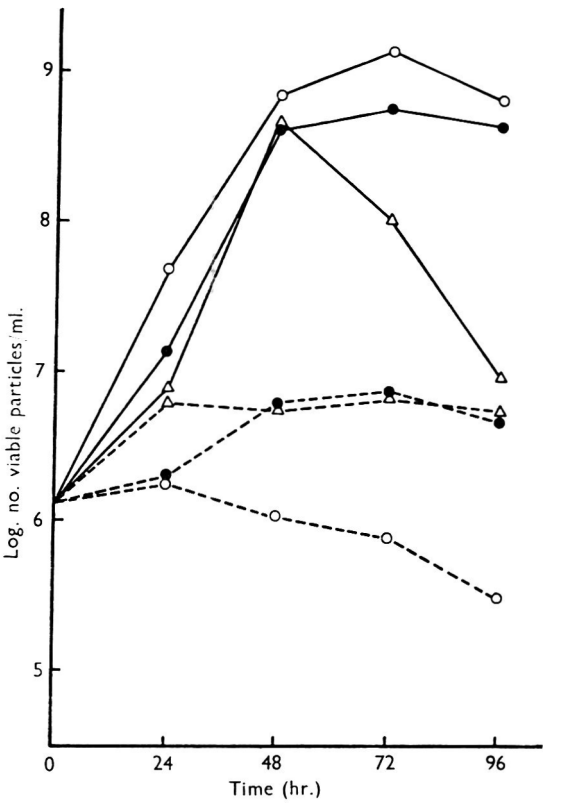

Fig. 4

Fig. 3. The growth of *Mycoplasma laidlawii* in partially defined medium containing different amounts of potassium chloride + 10 % (v/v) human serum.

Fig. 4. Requirement of glucose for growth of *Mycoplasma laidlawii*. The partially defined medium contained: 10 % (v/v) whole human serum (●); equiv. 10 % (v/v) human serum dialysed (○); 1 % (w/v) bovine serum albumin fraction V (△). —; with 0·75 % (w/v) glucose. – – –, without glucose.

sodium phosphate were omitted from the minimal medium (Table 2). Addition to the minimal medium of several inorganic salts known to be required by micro-organisms in trace amounts failed to improve growth. The salts tested were:

$Fe(NH_4)_2(SO_4)_2.6H_2O$; $MnSO_4.4H_2O$; $ZnSO_4.7H_2O$;
$CuSO_4.5H_2O$; $(NH_4)_6Mo_7O_{24}.4H_2O$; H_3BO_3.

Growth of *M. laidlawii* was inhibited by $Fe(NH_4)_2(SO_4)_2.6H_2O$ at concentrations of 10 μg./ml. or higher.

Carbohydrate requirement

Glucose was essential for the growth of *Mycoplasma laidlawii* in all the experimental media used in the present work (Fig. 4). The requirement for glucose, when tested in the albumin-containing medium, could be completely replaced by maltose, but not by galactose, fructose, mannose, lactose or sucrose.

Amino acid requirements

Casamino acids served as the main source of amino acids in the partially defined medium of Razin & Knight (1960*a*). Small amounts of amino acids were also supplied by the serum component of the medium. To eliminate the latter source of free amino acids, the serum was thoroughly dialysed. Dialysis did not affect to any significant degree the growth-promoting activity of the serum. Replacement of Casamino acids by known amino acid mixtures was then tried with the partially defined medium, which contained 10% (v/v) dialysed human serum. Thirteen different amino acid mixtures were tested. Twelve mixtures were compounded according to various authors who had used them in defined media for tissue cultures or microorganisms. One amino acid mixture was compounded according to an analysis of Casamino acids kindly provided by Difco Laboratories. Several of the tested amino acid mixtures enabled good growth of the test organism, but none equalled Casamino acids.

The mixture which provided best growth was then chosen for the analysis of the amino acid requirements. By a single omission technique, cystine and isoleucine were found to be essential for the growth of *Mycoplasma laidlawii*. Amino acids were now added one by one to cystine and isoleucine, and the resulting mixtures tested for their effect on the growth of *M. laidlawii*. Good growth could be obtained only with mixtures containing at least 15 amino acids. As most of these amino acids were interchangeable, it was suggested that mixtures containing a small number of amino acids were unable to promote growth not only because of the lack of essential amino acids, but also because of an insufficient amount of nitrogen in the medium. The effect of the addition of an ammonium salt was therefore tested. Ammonium sulphate or ammonium chloride markedly improved growth when incorporated into media containing various amino acid mixtures (Fig. 5). Potassium nitrate had no such effect.

The addition to the dialysed serum medium of $(NH_4)_2SO_4$ together with a mixture of 11 amino acids (the first eleven mentioned in Table 2) enabled good growth of *Mycoplasma laidlawii* equivalent to that obtained with Casamino acids (Fig. 6). Of the eleven amino acids included in this medium only cystine, isoleucine, glutamine and asparagine were shown to be definite growth requirements. The omission of any one of these 4 amino acids abolished or markedly decreased growth, whereas the omission of any of the other 7 affected growth slightly or not at all. However, growth in the presence of all 11 amino acids was always better than in the presence of cystine + isoleucine + glutamine + asparagine only.

No growth of *Mycoplasma laidlawii* took place with the above mentioned amino acid mixture when the dialysed serum was replaced by crystalline bovine plasma albumin or bovine serum albumin fraction V (Fig. 6). Growth appeared when

methionine was added, and was further improved by the addition of threonine. As seen in Fig. 6, growth of *M. laidlawii* in the medium containing a mixture of thirteen amino acids and 1% (w/v) bovine serum albumin fraction V was equal to that obtained with Casamino acids + dialysed serum. Experiments were carried out with a view to replacing the methionine required for growth of *M. laidlawii* in the albumin medium. The addition to the medium of homocysteine together with 'C_1 unit' donors, such as glycine, serine, formate or choline, with folinic acid and vitamin B_{12} as co-factors, did not enable growth when methionine was omitted.

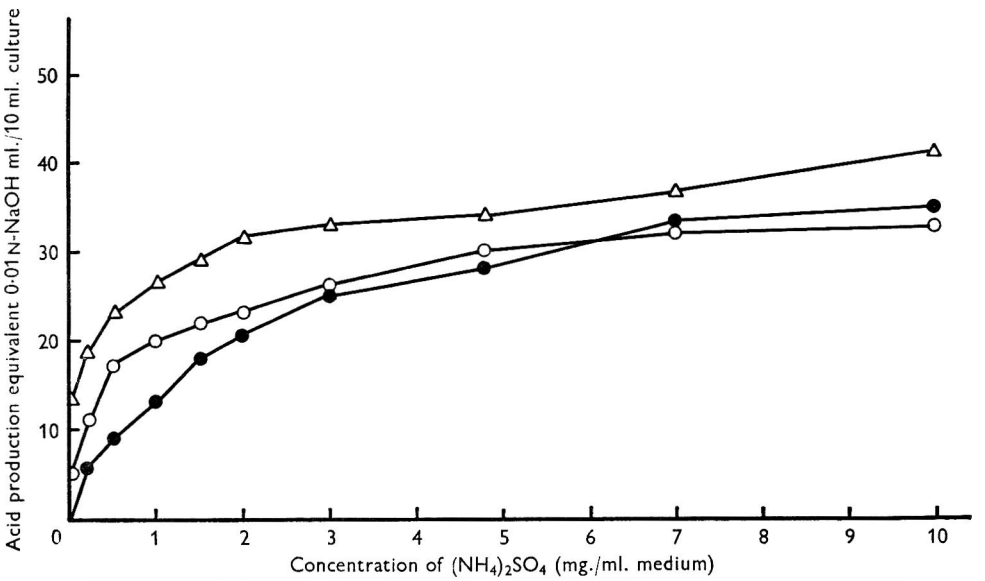

Fig. 5. The effect of ammonium sulphate on the growth of *Mycoplasma laidlawii*. The partially defined medium contained 10% (v/v) dialysed human serum and 10 amino acids (the first ten mentioned in Table 2; ●); 15 amino acids (glycine, DL-leucine, L-tyrosine, L-histidine, L-arginine, DL-valine, DL-isoleucine, L-tryptophan, DL-phenylalanine, DL-aspartic acid, L-lysine, L-glutamic acid, DL-methionine, L-cystine, DL-threonine; ○); 21 amino acids (the previous mixture + L-hydroxyproline, DL-serme, L-cysteine, DL-alanine, L-glutamine, L-asparagine; △).

Amino acid metabolism

To establish the most suitable conditions for testing amino acid metabolism of non-multiplying suspensions of *Mycoplasma laidlawii* manometric experiments with glucose as substrate were made. Glucose is known to be oxidized by *M. laidlawii* (Tourtellotte & Jacobs, 1960; Neimark & Pickett, 1960). It was found that even one washing of the organisms with 0·067 M-phosphate buffer (pH 7·6) markedly decreased their oxidative activity, while a second washing abolished it completely. As the endogenous oxygen uptake by unwashed organisms was very low, unwashed organisms were used for all subsequent experiments. The pH value of the phosphate buffer used in the reaction vessels had a marked influence on glucose oxidation by the suspensions. No oxygen uptake was noted at pH 6·0, low values were obtained at pH 7·0 and the highest values at pH 7·8 to 8·4. Consequently 0·067 M-phosphate buffer (pH 7·8) was chosen for the following experiments.

Twenty-two amino acids (glycine, DL-alanine, DL-leucine, DL-isoleucine, L-tyrosine, DL-phenylalanine, L-tryptophan, L-proline, L-hydroxyproline, L-histidine, L-arginine, DL-serine, DL-valine, DL-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-lysine, DL-methionine, L-cystine, L-cysteine, DL-threonine) were tested for oxidation and degradation by suspensions of *M. laidlawii*. In repeated experiments no significant oxygen uptake, substrate disappearance, or ammonia production was observed with any of these amino acids. Experiments were also made with strict adherence to the procedure described by Smith (1955) for demonstrating amino acid degradation by *Mycoplasma hominis*. The results of these experiments, carried out under aerobic and under anaerobic conditions and in the

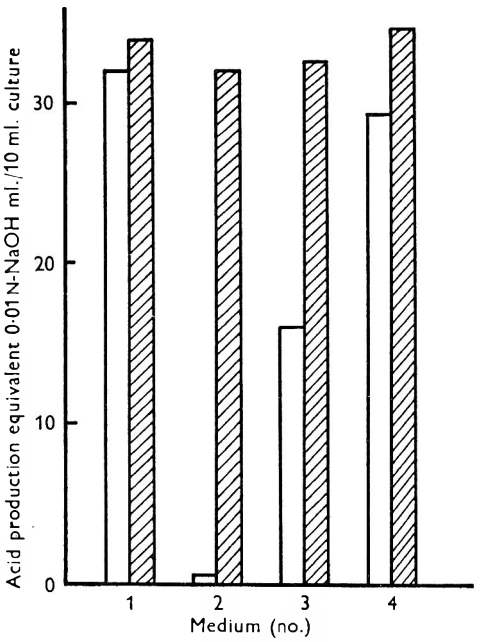

Fig. 6

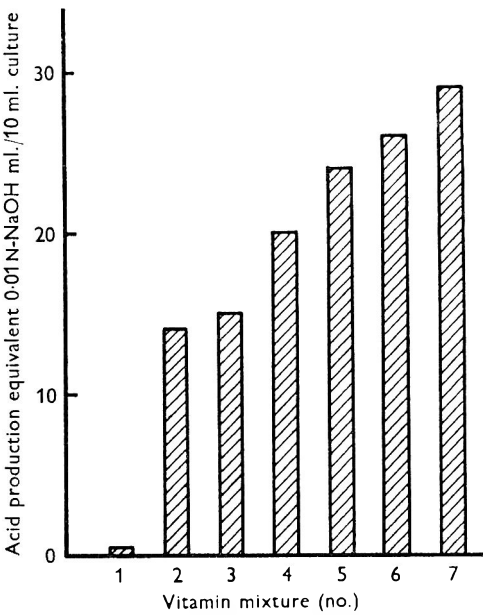

Fig. 7

Fig. 6. Growth of *Mycoplasma laidlawii* with Casamino acids or known amino acid mixtures. The partially defined medium contained 0·7 % (w/v) $(NH_4)_2SO_4$ + 10 % (v/v) dialysed serum or 1 % (w/v) bovine serum albumin fraction V + : (1) Casamino acids; (2) 11 amino acids (the first acids mentioned in Table 2); (3) the same as (2) + 0·2 mg. DL-methionine/ml.; (4) the same as (3) + 0·4 mg. DL-threonine/ml. □, bovine serum albumin (1 % w/v); ▨, dialysed human serum (10 % w/v).

Fig. 7. Growth of *Mycoplasma laidlawii* with various vitamin mixtures. The minimal medium (Table 2) contained: (1) riboflavin alone; (2) riboflavin + nicotinic acid; (3) as (2) + folinic acid; (4) as (3) + pyridoxine HCl; (5) as (4) + pyridoxal HCl; (6) as (5) + thiamine; (7) as (6) + nicotinamide, pantothenic acid (Ca salt), folic acid, choline-HCl, *m*-inositol. The final concentration of each vitamin was 2·5 μg./ml. medium, except folinic acid which was 1 μg./ml. medium.

presence of 10^{-5} M-pyridoxal phosphate were likewise negative. The addition of 5 μmole glucose to each of the reaction vessels did not promote degradation of any of the amino acids tested. A single experiment to test transaminase activity in a cell-free extract of *M. laidlawii* also gave negative results.

To make sure that our experimental conditions were suitable for amino acid degradation by Mycoplasma organisms a simultaneous experiment was carried out with *Mycoplasma laidlawii* and *M. hominis*, the latter having been found by Smith (1960) to degrade several amino acids. The following amino acids were tested: L-arginine, L-glutamine, L-glutamic acid, DL-aspartic acid, L-alanine. *M. laidlawii* did not attack any of these substrates, whereas *M. hominis* degraded arginine completely to citrulline and ornithine, and glutamine to glutamic acid (Table 1). This degradation was not accompanied by any significant oxygen uptake. The three other amino acids tested were not attacked by *M. hominis*.

Table 1. *Degradation of arginine and glutamine by resting* Mycoplasma hominis *organisms*

Each Warburg vessel contained 10 μmole substrate, 1 ml. cell suspension (equiv. 1 mg. N), 1 ml. 0·067 M-phosphate buffer (pH 7·8) 0·1 ml. 1×10^{-4} M-pyridoxal phosphate; total vol. 3·2 ml. In the centre well was 0·2 ml. of 15 % (w/v) KOH. Incubation was in air at 30° for 3 hr. The amount of substrates and degradation products was determined chromatographically at the end of the incubation period.

Substrate	Amount of substrate and degradation products at the end of the incubation period (μmole)				
	Arginine	Citrulline	Ornithine	Glutamine	Glutamic acid
L-Arginine	0	0·75	9·0	—	—
L-Glutamine	—	—	—	5·4	4·1

Vitamin requirements

The requirements of *Mycoplasma laidlawii* for nicotinic acid, riboflavin, folinic acid and pyridoxine or pyridoxal could be demonstrated in all the experimental media tested. In some experiments the omission of thiamine from the growth medium decreased growth to some extent. A mixture of the above mentioned six vitamins enabled growth of *M. laidlawii* comparable with that obtained with the full mixture of ten vitamins included in the partially defined medium (Fig. 7).

Nicotinamide, diphosphopyridine nucleotide or triphosphopyridine nucleotide were much less effective in growth-promotion than nicotinic acid, whereas flavin-adenine dinucleotide, pyridoxal phosphate and thiamine pyrophosphate were fully capable of replacing the entire requirement for the corresponding parent vitamins. The addition to the minimal medium (Table 2) of various vitamins and growth factors, such as vitamin B_{12}, biotin, *p*-aminobenzoic acid, thioctic acid or putrescine, did not improve the growth of *M. laidlawii*.

The minimal medium

The determination of inorganic ion, amino acid, carbohydrate, vitamin, nucleic acid precursor (Razin, 1962*b*) and albumin requirements, permitted the composition of a minimal medium, suitable for growth of *Mycoplasma laidlawii*. The ingredients of this medium are presented in Table 2. The extent of growth of the test organism in this minimal medium was comparable to that obtained in earlier serum-containing media (Fig. 8). The minimal medium was also found suitable for serial transfers of *M. laidlawii*. More than ten transfers were made in this medium without any reduction in growth. Sodium acetate, sodium lactate, glycerol and potassium

citrate have been reported to promote growth of various Mycoplasma organisms (Smith & Lynn, 1958; Rodwell & Abbot, 1961; Dr M. Shifrine, personal communication). These substances did not improve growth of *M. laidlawii* when added in various concentrations and mixtures to the minimal medium; lactate and citrate inhibited growth at concentrations higher than 0·5 mg./ml. The development of the minimal medium enabled a more direct approach to the examination of the cholesterol requirement of saprophytic Mycoplasma; no cholesterol was detected in this medium. Lipid extraction or dialysis of the serum albumin included in the medium did not affect growth of *M. laidlawii*. Cholesterol, when added to the minimal medium, did not improve growth.

Table 2. *The ingredients of the minimal medium for* Mycoplasma laidlawii

Inorganic salts (g./l.)		Amino acids (mg./l.)	
NaCl	6·8	L-Cystine	30
$(NH_4)_2SO_4$	7·0	DL-Isoleucine	600
2-Amino-2-hydroxymethylpropane-1:3-diol ('tris')	6·0	L-Glutamine	100
KCl	0·4	L-Asparagine	200
$MgSO_4.7H_2O$	0·2	Glycine	100
Na_2HPO_4	0·1	DL-Alanine	200
		DL-Phenylalanine	400
		L-Tyrosine	50
Vitamins (mg./l.)		L-Histidine-HCl	150
Nicotinic acid	2·5	DL-Leucine	800
Riboflavin	2·5	L-Arginine-HCl	200
Folinic acid	1·0	DL-Methionine	200
Pyridoxine-HCl	2·5	DL-Threonine	400
Pyridoxal-HCl	2·5		
Thiamine	2·5		
Nucleosides (mg./l.)		Miscellaneous	
Adenosine	100	Glucose:	7·5 g./l.
Guanosine	100	Bovine serum albumin fraction V:	10 g./l.
Cytidine	100	Penicillin G:	200 u./ml.

Medium finally adjusted to pH 8·3

Growth curves of Mycoplasma laidlawii *in the experimental media*

In most of the experiments described so far growth was measured indirectly by the titration of acid formed by the organisms during growth. Experiments with the viable count technique were therefore made to study more directly the rate and extent of growth of *Mycoplasma laidlawii* in the experimental media used. The growth curves presented in Fig. 8 show that growth obtained with the partially defined medium, containing either whole or dialysed serum, was comparable to that obtained in the complex Edward medium. Replacement of the serum by serum albumin fraction V or by crystallized albumin decreased growth to some extent. Replacement of the serum component by activated charcoal did not promote growth, but the inoculum survived for about 48 hr. and then decreased. Bacto-PPLO serum fraction (Smith & Morton, 1951) was much inferior to serum when tested in the partially defined medium. No growth of *M. laidlawii* took place in this medium when it contained less than 3% (v/v) of Bacto-PPLO serum fraction; growth with 10% (v/v) Bacto-PPLO serum fraction was still less to that obtained with serum (Fig. 8).

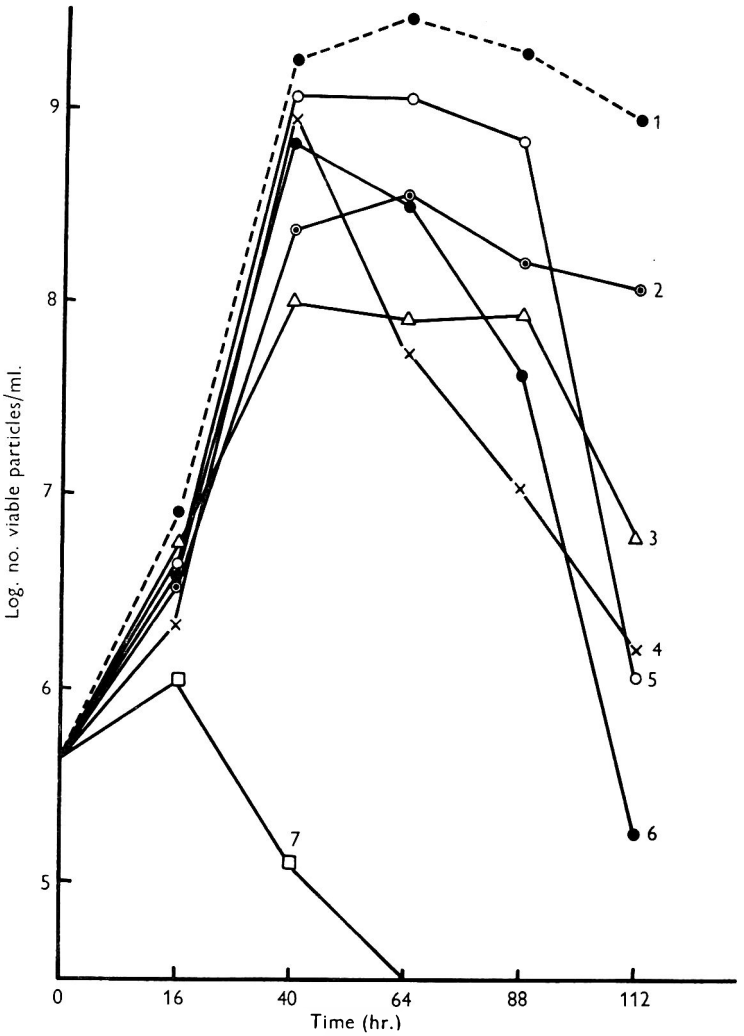

Fig. 8. Growth of *Mycoplasma laidlawii* in various experimental media. 1 = Edward medium; 2 = the partially defined medium + Casamino acids + 10 % (v/v) PPLO serum fraction; 3 = minimal medium + 1 % (w/v) crystallized bovine plasma albumin; 4 = minimal medium + 1 % (w/v) bovine serum albumin fraction V; 5 = minimal medium + 10 % (v/v) dialysed human serum; 6 = minimal medium + 10 % (v/v) whole human serum; 7 = minimal medium + 1 % (w/v) charcoal.

DISCUSSION

The minimal medium developed in the present work may not reflect the minimal growth requirements of *Mycoplasma laidlawii* in the strict sense; nevertheless it enabled a better analysis of the nutritional requirements of this organism than heretofore. The rate and extent of growth of *M. laidlawii* in this minimal medium exceeded that obtained in the less well-defined medium of Razin & Knight (1960*a*). The demonstration of the requirements for phosphate, Mg^{++} and K^{+} was to be expected, as these are common growth requirements of micro-organisms (Snell,

1951). The growth response of *M. laidlawii* to the concentration of NaCl (Fig. 2) conforms with the finding that Mycoplasma organisms can multiply only within very restricted tonicity values of the growth medium and require an optimal osmotic pressure of about 10 atmospheres (Rodwell, 1956; Adler & Shifrine, 1960; Leach, 1962). Sucrose only partially replaced the NaCl requirement; growth in the presence of sucrose was inferior to that with equimolar concentrations of NaCl. Similar results were obtained by Leach (1962, Table 4). Spermine, which enabled growth of the obligate halophile *Achromobacter fischerii* in a hypotonic NaCl-deficient medium (Mager, 1959) did not enable growth of *M. laidlawii* when NaCl was omitted from our medium. The failure of spermine to protect growth of Mycoplasma in the hypotonic medium is apparently due to growth inhibitory properties of this polyamine. The antibacterial effect of spermine was most pronounced in alkaline media (Rozansky, Bachrach & Grossowicz, 1954). This explains the marked growth inhibition of *M. laidlawii* by relatively low concentrations of spermine in the minimal medium, which has a pH value of 8·3. However, spermine did inhibit lysis of *M. laidlawii* by alternate freezing and thawing (Razin & Argaman, 1962). Thus spermine protects the fragile Mycoplasma cells from lysis, but at the same concentration inhibits their multiplication in the growth medium.

A metabolizable carbohydrate was found to be indispensable for the growth of *Mycoplasma laidlawii* in the minimal medium. This finding may be correlated with the inability of this Mycoplasma strain to catabolize amino acids. The carbo hydrate included in the medium therefore served both as a source of carbon and of energy. Similar findings were reported by Rodwell (1960) for *Mycoplasma mycoides* var. *mycoides*. This Mycoplasma organism also required a metabolizable carbohydrate in the medium, and its amino acid catabolism was very limited, serine and threonine being the only amino acids which were degraded. Although *M. laidlawii* was reported by Edward (1954) and Freundt (1958) to be able to ferment fructose, no significant growth was obtained when glucose in the minimal medium was replaced by fructose. However, the fermentation studies of Edward and Freundt were carried out in rich complex media.

The Casamino acids included in the partially defined medium of Razin & Knight (1960*a*) were replaced by a mixture of 13 amino acids without affecting growth. Apparently not all amino acids included in this mixture were essential growth requirements; several amino acids could be omitted without decreasing the amount of growth. Methionine and threonine were required only when serum albumin was substituted for dialysed serum in the medium. This might have been due to the presence of sufficient amounts of these amino acids in the dialysed serum. Dialysed human serum, stored for long periods at 5°, was found to contain significant amounts of free amino acids liberated by proteolysis (Piez, Oyama, Levintow & Eagle, 1960). We therefore stored the dialysed serum at $-10°$ to minimize this proteolytic effect. Our findings indicate the ability of *Mycoplasma laidlawii* to utilize ammonium ion as a nitrogen source. The inability of this Mycoplasma to utilize nitrate for this purpose is in accordance with the findings of Kandler & Kandler (1955) and Freundt (1958), who were unable to demonstrate nitrate reduction by *M. laidlawii*.

Rather unexpected was our complete failure to show degradation of any amino acid by suspensions of *Mycoplasma laidlawii*. This could hardly be attributed to inadequate experimental conditions, since arginine and glutamine were degraded

by a culture of *Mycoplasma hominis* under identical conditions. It seems that amino acids are not good energy and carbon sources for any of the Mycoplasma strains tested so far. Even with Mycoplasma strains which are incapable of utilizing carbohydrates, amino acids cannot serve as the sole energy and carbon source, since amino acid metabolism of these strains is predominantly anabolic in nature (Smith, 1960).

Of the vitamins required by *Mycoplasma laidlawii*, nicotinic acid, riboflavin and thiamine were required also by *M. mycoides* var. *mycoides* (Rodwell, 1960). The requirement for folinic acid could be met by the addition of thymidine to the growth medium (Razin, 1962*b*). The no more than partial replacement of the nicotinic acid requirement by nicotinamide is not yet explained. The function of the serum albumin included in the minimal medium has not yet been studied in detail. The question is whether the albumin acts as a nutrient or merely as an adsorbent of growth inhibitors, such as unsaturated fatty acids (Davis & Dubos, 1947). It seems that neutralization of toxic agents is not the sole function of the albumin since in the minimal medium it was not replaced by charcoal. Albumin might act as a carrier of essential nutrients (Eagle & Piez, 1960) or might provide an adequate physical environment for growth of the plastic Mycoplasma organisms (Salzman, 1961).

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Lysis of Mycoplasma, Bacterial Protoplasts, Spheroplasts and L-forms by Various Agents

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SUMMARY

The susceptibility to lysis of several Mycoplasma organisms, bacterial protoplasts, spheroplasts, L-forms and intact bacteria was compared. The Mycoplasma and L-forms were much more resistant to lysis by osmotic shock and to alternate freezing and thawing than were the bacterial protoplasts and spheroplasts. Like bacterial protoplasts, the mycoplasmas were very sensitive to lysis by surface-active substances, primary alcohols and alkali; bacterial spheroplasts and L-forms were less sensitive to lysis by these agents. All the organisms tested, except intact bacteria, showed various degrees of sensitivity to lysis by pancreatic lipase. The mycoplasmas and the L-forms were completely resistant to the lytic action of lysozyme. The mycoplasmas differed from all other micro-organisms tested in being sensitive to lysis by digitonin and saponin. This sensitivity to digitonin was considerably smaller when the organism was grown in a cholesterol-free defined medium. The structure and properties of the Mycoplasma cell envelope are discussed on the basis of the present findings.

INTRODUCTION

One of the most peculiar features of the Mycoplasma organisms (pleuropneumonia-like organisms, PPLO) is their thin and plastic envelope. Evidence for the absence of a rigid cell wall has been furnished by electron microscope studies of whole mycoplasma cells and of ultrathin sections (Van Iterson & Ruys, 1960; Ruys & Van Iterson, 1961; Edwards & Fogh, 1960; Sharp 1960; Klieneberger-Nobel, 1962). Ultrathin sections of mycoplasma organisms showed the envelope to consist of two electron-dense outer layers and a transparent inner zone (Van Iterson & Ruys, 1960; Ruys & Van Iterson, 1961) resembling the 'unit membrane' which is of universal distribution in nature (Robertson, 1959). The thickness of the mycoplasma cell envelope was found to be $\approx$ 75 Å by Van Iterson & Ruys (1960), similar to that of the bacterial plasma membrane (Thorsson & Weibull, 1958; van Iterson, 1961; Imaeda & Convit, 1962). Lack of the 'mucopeptide' polymer in Mycoplasma (Kandler & Zehender, 1957; Plackett, 1959; Razin & Argaman, 1961) further supported the assumption that Mycoplasma has no cell wall and is limited by a membrane which resembles the plasma membrane of bacterial protoplasts. Very little is known as yet about the chemical composition of the Mycoplasma envelope. The presence of a cardiolipin-like compound in the envelope of *Mycoplasma mycoides* var. *mycoides* was suggested by Rodwell & Abbot (1961). Complex phosphatidic

acid, resembling cardiolipin, was found to be the major lipid component of the bacterial plasma membrane (Weibull & Bergström, 1958; Gilby, Few & McQuillen, 1958). Carotenoid pigments were found in the 'membrane fraction' of *Mycoplasma laidlawii* (Rothblat & Smith, 1961), as in the plasma membrane of *Micrococcus lysodeikticus* (Gilby *et al.* 1958). Rothblat & Smith (1961) found cholesterol or derivatives of it in the 'membrane fraction' of several Mycoplasma strains. No cholesterol has so far been found in any of the bacterial plasma membranes studied (Weibull & Bergström, 1958; Gilby *et al.* 1958).

The apparent similarity between the Mycoplasma envelope and that of bacterial protoplasts prompted us to compare the susceptibility of these organisms to lysis by various physical and chemical agents. Bacterial spheroplasts and stable L-forms containing defective cell walls (Klieneberger-Nobel, 1960) were also examined.

METHODS

Organisms. *Mycoplasma laidlawii* strain A (PG 8) was obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *Mycoplasma mycoides* var. *capri* and the stable L-form of *Streptobacillus moniliformis* were kindly provided by Dr E. Klieneberger-Nobel (The Lister Institute of Preventive Medicine, London). *Mycoplasma hominis* no. 23 was isolated in our laboratory from a case of vaginitis. *Micrococcus lysodeikticus* and *Escherichia coli* strain B were obtained from the collection of the Department.

Media and organism suspensions. *Mycoplasma laidlawii* and *M. mycoides* var. *capri* were grown in a modified liquid Edward medium (Razin & Oliver, 1961) containing 2% (v/v) Bacto PPLO serum fraction. *Mycoplasma hominis* and the L-form of *Streptobacillus moniliformis* were grown in the same medium, but with 15% (v/v) inactivated (pooled) human serum instead of PPLO serum fraction. Growth was carried out in rotated Erlenmeyer flasks for 24–48 hr. at 37°. The organisms were collected in a Sharples supercentrifuge and resuspended in de-ionized water or in M-sucrose + 0·05M-NaCl solution, referred to below as the sucrose medium. *Micrococcus lysodeikticus* was grown on brain-heart infusion agar (Difco) for 18 hr. at 37°, collected, washed three times in de-ionized water and resuspended in de-ionized water or in the sucrose medium.

Preparation of protoplasts. Protoplasts of *Micrococcus lysodeikticus* were prepared by the action on the washed organisms (at equiv. 10 mg. dry wt./ml.) of crystalline lysozyme (100 μg./ml.) in the sucrose medium (Gilby & Few, 1960*a*). The quantitative conversion of the bacteria into protoplasts was usually accomplished after incubation at 37° for 30–45 min. The resulting protoplasts were centrifuged down and after the supernatant fluid was decanted, were gently resuspended in the sucrose medium.

Preparation of spheroplasts. The method described by Lederberg (1956) was used. *Escherichia coli* B was grown in 1 l. nutrient broth (Difco) at 37° for 18 hr. The broth culture was then transferred to 3 l. fresh nutrient broth containing 20% (w/v) sucrose + 0·2% (w/v) $MgSO_4 . 7H_2O$ + 1000 units crystalline penicillin G/ml. Incubation was continued at 37°, and the formation of spheroplasts followed by phase-contrast microscopy. The quantitative conversion of the bacilli into spheroplasts usually took place after 4–5 hr. of incubation. The spheroplasts were sedimented by

centrifugation at 7000 *g* for 15 min. and resuspended in 20 % (w/v) sucrose + 0·2 % (w/v) $MgSO_4.7H_2O$ solution.

Chemicals. Spermine tetrahydrochloride was the product of Hoffmann La Roche and Co. Ltd. (Basle, Switzerland); protamine sulphate was obtained from L. Light & Co. Ltd. (Colnbrook, Bucks., England) poly-L-lysine (n-34) was a gift of Professor E. Katchalski (The Weizmann Institute of Science, Rehovoth, Israel). Sodium lauryl sulphate (U.S.P.) was the product of Amend Drug and Chemical Co. (New York 10, N.Y., U.S.A.). Cetyltrimethylammonium bromide (pure: CTAB) was obtained from Hopkin & Williams Ltd. (London, E.C. 1). Sodium deoxycholate and sodium taurocholate were the products of Difco Laboratories, Inc., Detroit 1, Michigan, U.S.A. 'Tween' 80, U.S.P. was bought from the City Chemical Co. (New York) and 'Triton' WR-1339 from Winthrop-Stearns Inc. (New York 18, N.Y., U.S.A.). Ethanol, *n*-propanol and *n*-butanol of A.R. quality were used without further purification. Digitonin was obtained from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and saponin from Hopkin & Williams Ltd. (London, E.C. 1). Lysozyme chloride (crystalline, egg white) was the product of L. Light & Co. Trypsin (2 × crystallized), lipase (bovine), bromelin and pepsin (1/20,000) were bought from Nutritional Biochemicals Corporation.

Examination of lysis

Tonicity changes. Test tubes contained 4 ml. of serial twofold dilutions of sucrose medium or of a 2 M-NaCl (in 0·01 M-phosphate buffer, pH 6·8) solution. Each test tube received 0·1 ml. suspension of test organism to an optical density (OD) of 0·3–0·4 at 500 mμ. Changes in optical density were measured after 30 min. incubation at room temperature in a 'Unicam SP 500' spectrophotometer. The effect of the substitution of other solutes for the sucrose or NaCl in the suspending medium was tested by the method of Mitchell & Moyle (1956*a*). Samples (0·1 ml.) of suspensions of the organisms in the sucrose medium were added to 4 ml. of 1·5 M solution (in 0·01 M-phosphate buffer, pH 6·8) of the solute to be tested. The rate of lysis was measured by optical density changes at room temperature during short time intervals.

Alternate freezing and thawing. Suspensions of the organisms were made in dilutions of the sucrose medium or in de-ionized water. The suspensions were dispensed into test tubes in quantities of 4 ml. and frozen in an ethanol bath at −24°. When frozen, the test tubes were transferred for thawing to a water bath at 40°. This procedure was repeated as many times as required and the optical density measured after each freezing and thawing.

Heating. Suspensions of organisms were made in sucrose medium, dispensed in 4 ml. quantities in test tubes and heated for 10 min. at 40°, 50°, 60°, 70° or 80°. The optical density of the suspensions was measured as described above.

Chemicals. Solutions of the chemicals to be tested were prepared in 4 ml. quantities of sucrose medium or in de-ionized water. Each test tube received 0·1 ml. of suspension of organism in sucrose medium or de-ionized water. Changes in the optical densities of the suspensions were determined after 30 min. of incubation at room temperature. In some experiments where ionic detergents were tested, the organisms were pre-treated with uranyl nitrate according to Gilby & Few

(1960*a*). The organisms were suspended in the usual sucrose medium, containing different concentrations of uranyl nitrate. After 30 min. incubation at room temperature the organisms were centrifuged down, resuspended in sucrose medium and recentrifuged. Finally, they were resuspended in sucrose medium and lysis with the detergents was tested as above.

Enzymes. The sensitivity of the organisms to the lytic action of lysozyme was tested on washed organisms suspended in 0·067 M-phosphate buffer (pH 6·8; Smolelis & Hartsell, 1949) or on organisms pre-treated by heating and changing pH from 3·5 to 10·5 (Grula & Hartsell, 1957), or treatment by freezing and thawing (Kohn, 1960), trypsinization (Becker & Hartsell, 1954), treatment with ethylenediaminetetra-acetate (EDTA; Repaske, 1956).

The effect of proteolytic and lipolytic enzymes was tested on organisms suspended in 0·067 M-phosphate buffer (pH 7·0), or 0·01 M-phosphate buffer (pH 8·0) containing 0·002 M-Mg^{++}, Ca^{++} and Mn^{++} as chloride. For comparative experiments with bacterial protoplasts and spheroplasts, sucrose was added to the buffer to final concentration of 1M. The test tubes thus contained 3·5 ml. enzyme solution in buffer and 0·5 ml. suspension of organisms. Parallel experiments were carried out with organisms pre-treated by heating at 70° for 15 min. The test tubes were incubated at 37° and optical density changes determined at various time intervals.

Agglutination by polyamines. Agglutination of the organisms by polyamines was tested in de-ionized water according to the method of Razin & Rozansky (1959).

RESULTS

Lysis by decreasing the tonicity of the medium

The effect on the test organisms of decreasing the tonicity of the medium was measured by observing the optical density changes of suspensions prepared in sucrose or NaCl solutions (Fig. 1). The protoplasts of *Micrococcus lysodeikticus* were very sensitive to decreases in tonicity and underwent almost complete lysis at 0·25 M-sucrose. The spheroplasts of *Escherichia coli* were less sensitive to lysis than the protoplasts; decreases in the optical density of their suspensions appeared only at concentrations of sucrose less than 0·12M. The Mycoplasma organisms observed and the L-form of *Streptobacillus moniliformis* were not lysed to any significant degree even by transfer from M-sucrose solution to de-ionized water. The increase in the optical density of the Mycoplasma suspensions at higher sucrose concentrations might have been due to the shrinkage of the plastic cells in the hypertonic medium, while the decrease in optical density at the highest sucrose concentrations was apparently caused by depression of the light scattering of the organisms because of the high refractive index of the concentrated sucrose solution (Gilby & Few, 1959). The decrease of the optical density of bacterial suspensions at increasing sucrose concentrations is clearly demonstrated in the case of intact *Micrococcus lysodeikticus* (Fig. 1). The results were similar to those described above with media which contained different amounts of sodium chloride. Attempts at assessment by phase contrast microscopy (maximal magnification ×1250) of changes in cell volume of *Mycoplasma laidlawii* suspended in media of various tonicity failed owing to the very small dimensions of the Mycoplasma organisms.

The nature of the solute used for obtaining the required tonicity of the suspending

medium was of great importance. Solutes capable of penetrating bacterial protoplasts (e.g. glycerol, D-ribose) were unsuitable for use as osmotic stabilizers (Mitchell & Moyle, 1956*a*). Replacement of the sucrose in our medium by other sugars or by glycerol was therefore tried. It may be seen from Fig. 2 that the protoplasts of *Micrococcus lysodeikticus* and the spheroplasts of *Escherichia coli* underwent complete and immediate lysis when suspended in 1·5 M-glycerol solution. Lysis in 1·5 M-D-ribose solution was slower. *Mycoplasma laidlawii* and the L-form of *Streptobacillus*

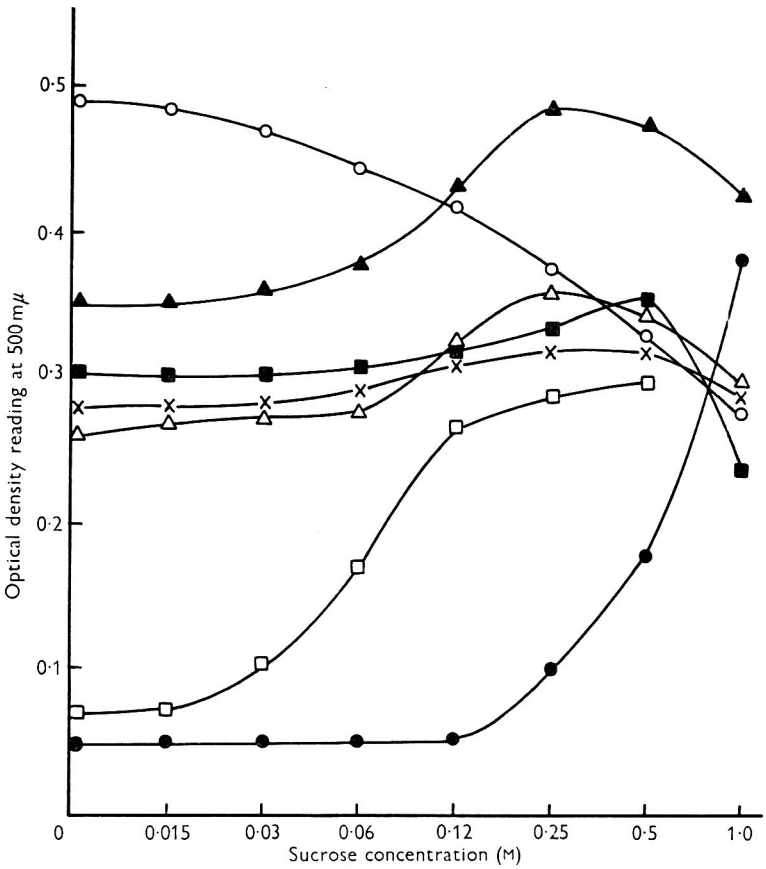

Fig. 1. The effect of the sucrose concentration in the suspension medium on the optical density of suspensions of *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); *Mycoplasma hominis* (■); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); *Micrococcus lysodeikticus* (○) and protoplasts of *M. lysodeikticus* (●). Optical density measured after 30 min. incubation at room temperature.

moniliformis were lysed to a certain degree in glycerol or D-ribose solution. No significant lysis of any of the organisms tested occurred when 1·5 M-glucose or fructose replaced sucrose in the suspending medium. Magnesium sulphate (0·2%, w/v) decreased the degree of lysis of the spheroplasts of *E. coli* in 1·5 M-glycerol or in de-ionized water by about one half. Spermine (10 μg./ml.) completely protected the spheroplasts from lysis in these media.

Lysis by alternate freezing and thawing

When suspended in the sucrose medium, Mycoplasma organisms, L-form of *Streptobacillus moniliformis*, spheroplasts of *Escherichia coli* and intact *Micrococcus lysodeikticus* were not lysed by alternate freezing and thawing. Ten transfers from

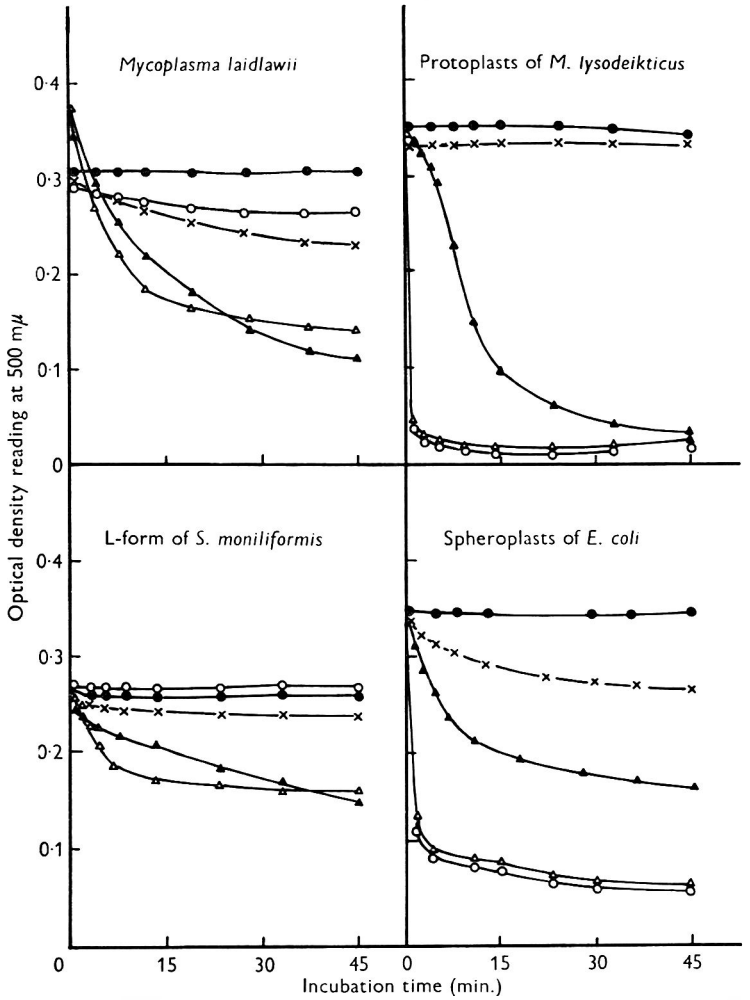

Fig. 2. Optical density changes of suspensions prepared in 1·5 M solutions (in 0·01 M-phosphate buffer, pH 6·8) of sucrose (●); glucose (×); glycerol (△); ribose (▲); 0·01 M-phosphate buffer (pH 6·8) alone (○). Incubation was carried out at room temperature.

−24° to +40° did not cause any significant decrease in the optical density of suspensions of these organisms, while protoplasts of *M. lysodeikticus* underwent almost complete lysis after several alternate freezings and thawings (Fig. 3). However, the Mycoplasma organisms became sensitive to lysis when the sucrose concentration in the medium was markedly decreased. The degree of lysis in the presence of 0·006 M-sucrose equalled that in de-ionized water, but even 0·06 M-sucrose had a significant protective effect (Fig. 3).

Spermine, known to protect some fragile organisms and organelles from lysis under unfavourable conditions (Tabor, Tabor & Rosenthal, 1961) was tested for such effect on Mycoplasma treated by freezing and thawing in de-ionized water. A concentration of 10 μg./ml. of this polyamine protected Mycoplasma organisms from lysis by ten alternate freezings and thawings, while 2 μg./ml. was insufficient. These results were obtained with both *Mycoplasma mycoides* var. *capri* and *M. laidlawii*. Spermine, protamine and poly-L-lysine agglutinated *M. mycoides* var.

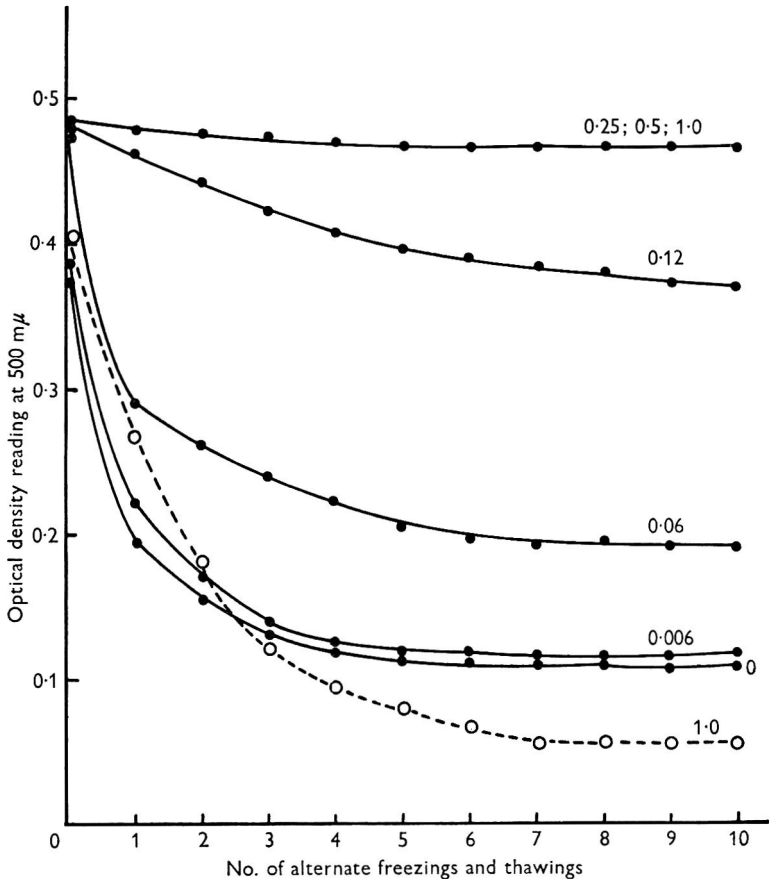

Fig. 3. Lysis of *Mycoplasma laidlawii* by alternate freezing and thawing in media containing different molar concentrations of sucrose (indicated in figure). The lysis curve of protoplasts of *Micrococcus lysodeikticus* in M-sucrose (O – – O) is included for comparison.

capri and *M. laidlawii* within a certain range of concentrations (spermine, 80–1250 μg./ml.; protamine, 80–5000 μg./ml.; polylysine, 80–320 μg./ml.). Intact *M. lysodeikticus* organisms were agglutinated by the same polyamine concentrations. The aggregates were formed within 1–2 hr. of incubation at 37°; they sedimented overnight, leaving the supernatant fluid clear.

Lysis by heating

Spheroplasts of *Aerobacter aerogenes* were found to lyse at temperatures of about 56–65° (Gebicki & James, 1960). Similar experiments with our test organisms suspended in the sucrose medium showed that the optical density of the suspension of *Micrococcus lysodeikticus* protoplasts decreased by about 50% after heating at 80° for 10 min. A decrease of about 30% in optical density was found with the Mycoplasma suspensions heated to the same temperature; the optical density of these suspensions began to decrease at 50°.

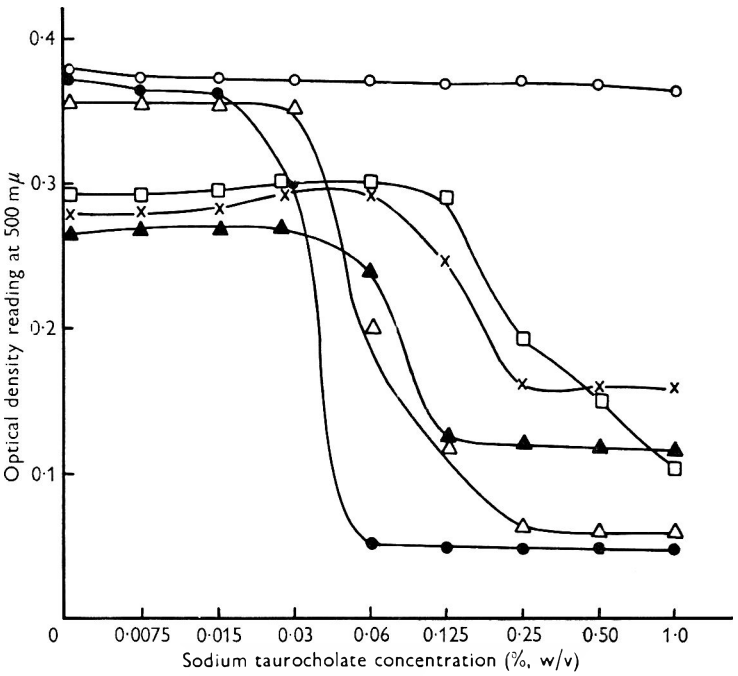

Fig. 4. Lysis by sodium taurocholate. *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); intact cells of *Micrococcus lysodeikticus* (○); protoplasts of *M. lysodeikticus* (●). The experiment was carried out in the sucrose medium and optical density was measured after 80 min. incubation at room temperature.

Lysis by treatment with surface active agents

The Mycoplasma organisms tested were sensitive to lysis by sodium deoxycholate and sodium taurocholate (Fig. 4). The Mycoplasma organisms were more sensitive to lysis by these agents than were the L-form of *Streptobacillus moniliformis* and spheroplasts of *Escherichia coli*. On the other hand the Mycoplasma organisms were more resistant to lysis than were protoplasts of *Micrococcus lysodeikticus*. Similar results were obtained with the anionic detergent sodium lauryl sulphate (Fig. 5*a*). The cationic detergent cetyltrimethylammonium bromide (CTAB) caused lysis of the bacterial protoplasts and of *Mycoplasma laidlawii* only within a narrow range of concentrations; at higher concentrations agglutination and precipitation of the

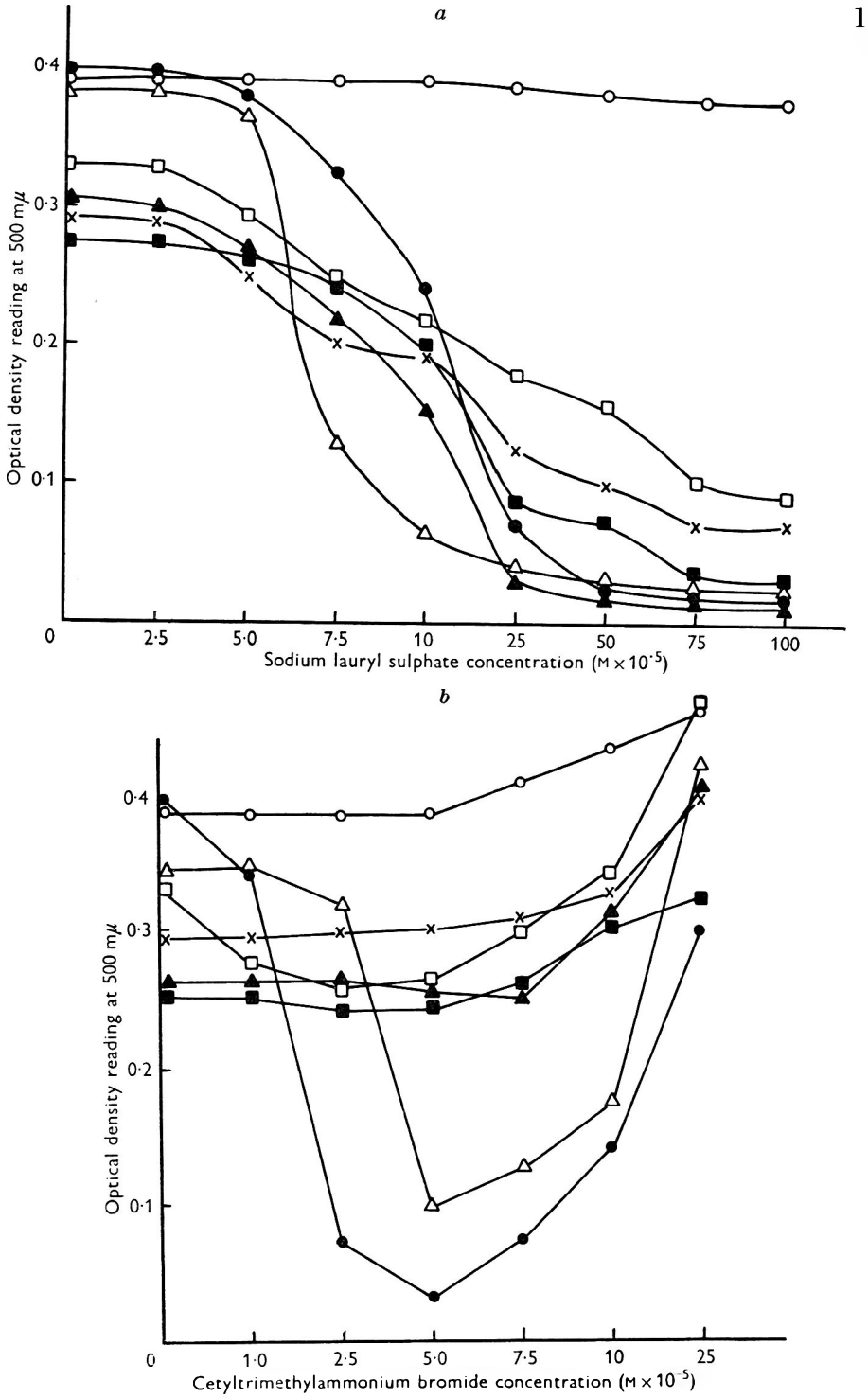

Fig. 5. Lysis by ionic detergents. (*a*) Sodium lauryl sulphate; (*b*) cetyltrimethylammonium bromide. *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); *Mycoplasma hominis* (■); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); intact *Micrococcus lysodeikticus* (○); protoplasts of *M. lysodeikticus* (●). The experiment was carried out in the sucrose medium and optical density was measured after incubation for 30 min. at room temperature.

organisms occurred and the optical density of their suspensions increased (Fig. 5*b*). The cationic detergent CTAB had no marked lytic effect on any of the other organisms tested. Pre-treatment of *M. laidlawii* organisms and *M. lysodeikticus* protoplasts with uranyl nitrate gave protection against lysis by anionic and by cationic detergents (Fig. 6).

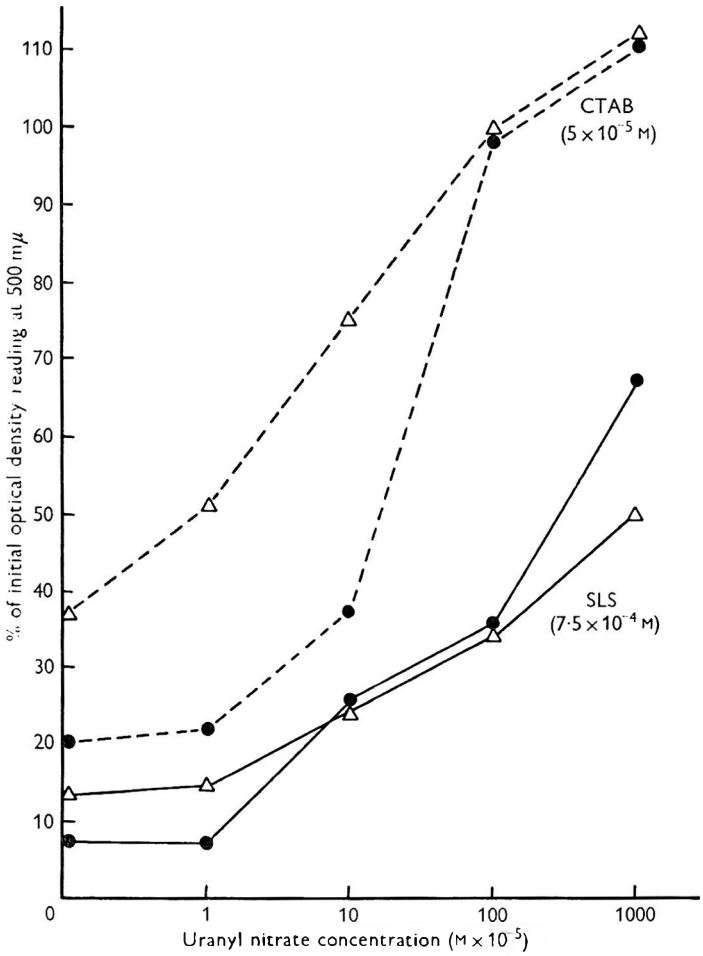

Fig. 6. Lysis by sodium lauryl sulphate (SLS) and cetyltrimethylammonium bromide (CTAB) of *Mycoplasma laidlawii* (△) and protoplasts of *Micrococcus lysodeikticus* (●) pretreated with different concentrations of uranyl nitrate. The experiment was carried out in the sucrose medium. Optical density was measured after incubation for 30 min. at room temperature and plotted as % optical density of suspensions before the addition of detergents.

The non-ionic detergents Tween 80 and Triton had a much lower lytic activity than had the ionic detergents, and caused no lysis when the organisms were suspended in the sucrose medium. Some lysis occurred when *Mycoplasma laidlawii* was suspended in de-ionized water. None of the other Mycoplasma organisms nor the L-form of *Streptobacillus miniliformis* were lysed by non-ionic detergents, even when suspended in de-ionized water.

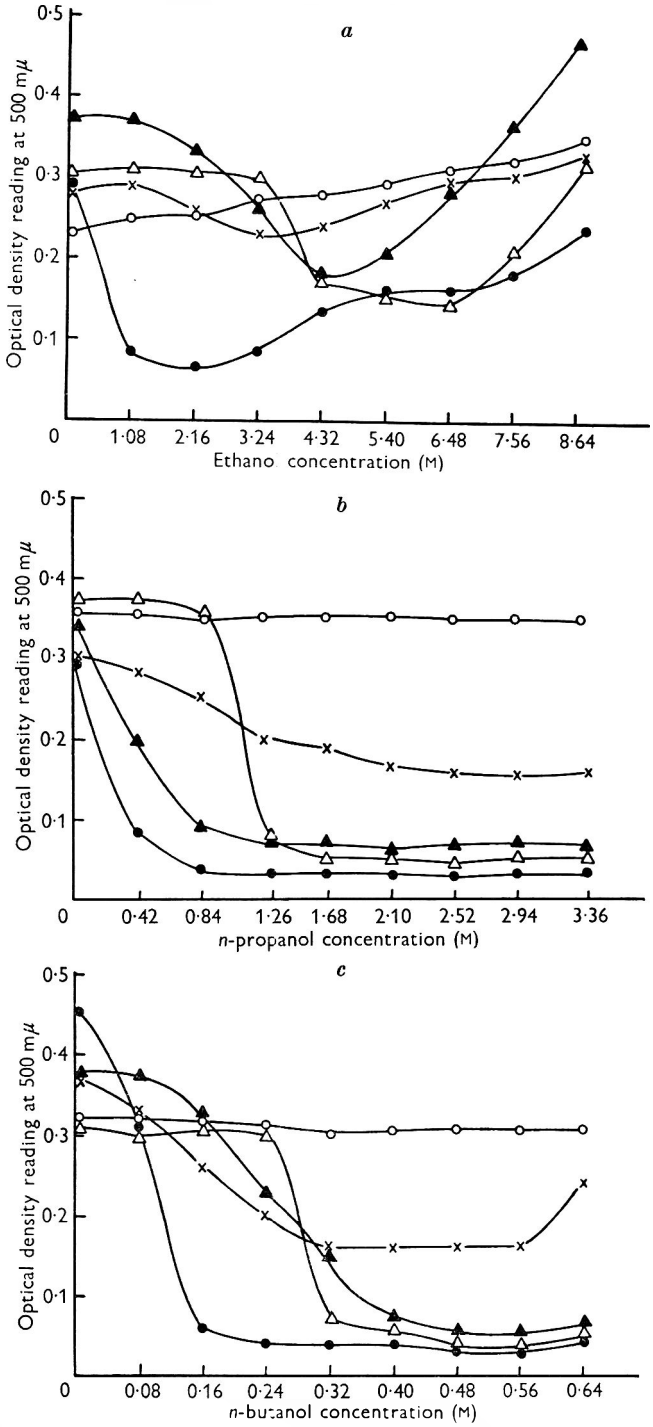

Fig. 7. Lysis by primary alcohols. (*a*) Ethanol; (*b*) *n*-propanol; (*c*) *n*-butanol. *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); L-form of *Streptobacillus moniliformis* (×); intact cells of *Micrococcus lysodeikticus* (○); protoplasts of *M. lysodeikticus* (●). The experiment was carried out in the sucrose medium and optical density was measured after incubation for 30 min. at room temperature.

Lysis by alcohols

The Mycoplasma protoplasts, like bacterial protoplasts, were very sensitive to lysis by primary alcohols (Fig. 7). On a molar basis the lytic effect of *n*-butanol was about four times greater than that of *n*-propanol and about ten times greater than that of ethanol. The increase in the optical density of the suspensions at high ethanol concentrations was apparently due to precipitation of the cellular components at these high alcohol concentrations. The L-form of *Streptobacillus moniliformis* was much more resistant to the lytic effects of alcohols than were the Mycoplasma organisms.

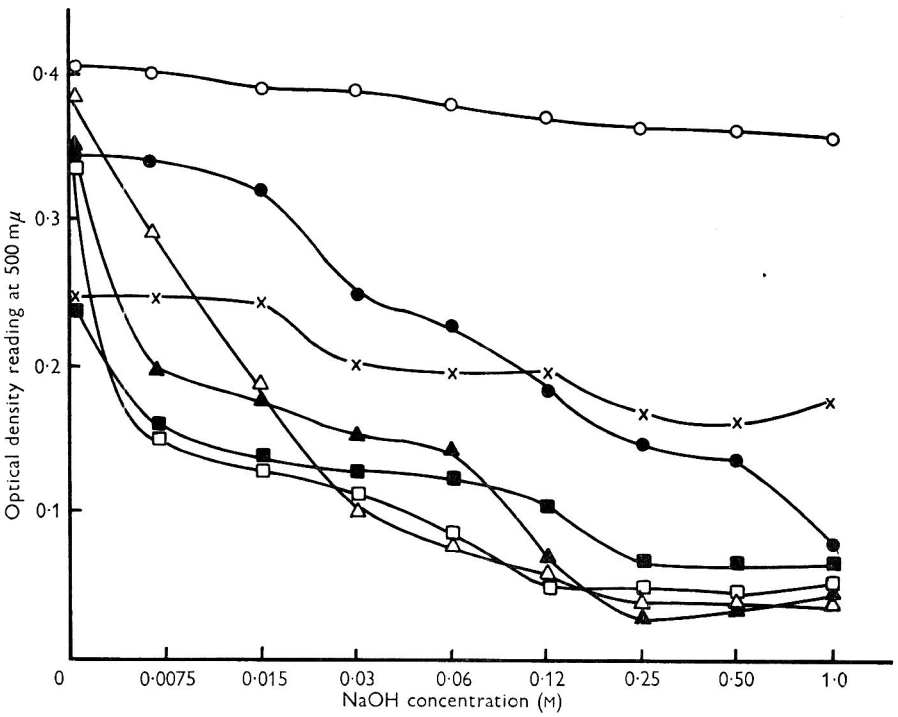

Fig. 8. Lysis by alkali. *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); *Mycoplasma hominis* (■); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); intact *Micrococcus lysodeikticus* (○); protoplasts of *M. lysodeikticus* (●). The experiment was carried out in the sucrose medium and the optical density measured after incubation for 30 min. at room temperature.

Lysis by alkali

All the organisms tested, except intact *Micrococcus lysodeikticus*, showed various degrees of sensitivity to lysis by NaOH. Mycoplasma organisms and spheroplasts of *Escherichia coli* showed the highest sensitivity; bacterial protoplasts and L-forms were less sensitive to lysis by alkali (Fig. 8). Hydrochloric acid did not cause lysis of any of the test organisms within the range of concentrations 0·03–5·0 M.

Lysis by digitonin and saponin

All the Mycoplasma strains tested showed a marked sensitivity to lysis by digitonin and saponin; all the other organisms tested were completely resistant. Human erythrocytes were more sensitive than Mycoplasma organisms to lysis by digitonin and saponin (Fig. 9). Washed digitonin-resistant organisms were not rendered sensitive to lysis by exposure to 0·01 mg. cholesterol/ml. in the sucrose medium for 3 hr. at 37°. *Mycoplasma laidlawii* organisms grown in a cholesterol-free defined medium (Razin & Cohen, 1962) were much more resistant to lysis by digitonin and saponin than were organisms grown in the cholesterol-containing Edward medium (Fig. 10).

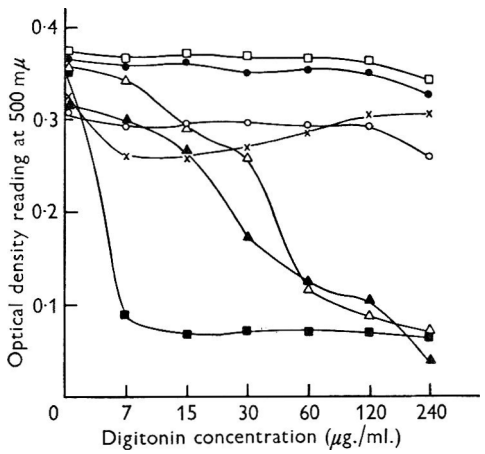

Fig. 9

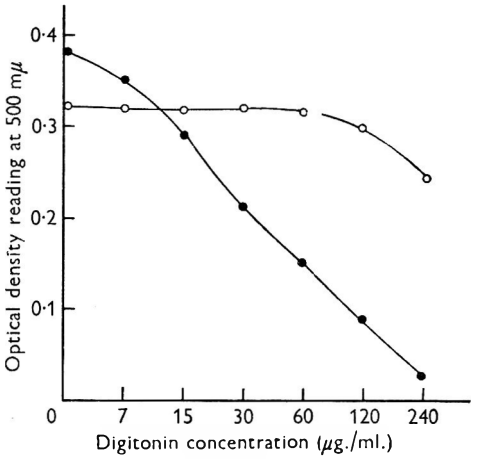

Fig. 10

Fig. 9. Lysis by digitonin. *Mycoplasma laidlawii* (△); *Mycoplasma mycoides* var. *capri* (▲); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); intact *Micrococcus lysodeikticus* (○); protoplasts of *M. lysodeikticus* (●); human erythrocytes (■). The experiment was carried out in the sucrose medium and the optical density measured after incubation for 30 min. at room temperature.

Fig. 10. Lysis by digitonin of *Mycoplasma laidlawii* grown in a cholesterol-free defined medium (Razin & Cohen, 1962; ○) or in the cholesterol-containing Edward medium (●). The experiment was carried out in de-ionized water and optical density measured after incubation for 30 min. at room temperature.

Lysis by enzymes

Lysozyme. This enzyme had no lytic effect on Mycoplasma organisms or on the L-form of *Streptobacillus moniliformis* by any of the methods tested.

Lipase. Crude pancreatic lipase caused lysis of all the Mycoplasma strains examined. The lytic effect was greater in 0·067 M-phosphate buffer (pH 7·0) than in 0·01 M-phosphate buffer (pH 8·0). Pancreatic lipase also caused lysis of bacterial protoplasts, spheroplasts and L-forms. Intact organisms of *Micrococcus lysodeikticus* and *Escherichia coli* were not lysed by this enzyme (Fig. 11).

Proteolytic enzymes. Trypsin had very little lytic effect on any fresh organism tested. A marked lytic effect was seen with the Mycoplasma organisms and spheroplasts of *Escherichia coli* when these were preheated at 70° for 15 min. The L-form

of *Streptobacillus moniliformis* was insensitive to lysis by trypsin even when pre-heated. Of the other proteolytic enzymes tested, bromelin, like trypsin, acted only on heated organisms; papain had no lytic activity.

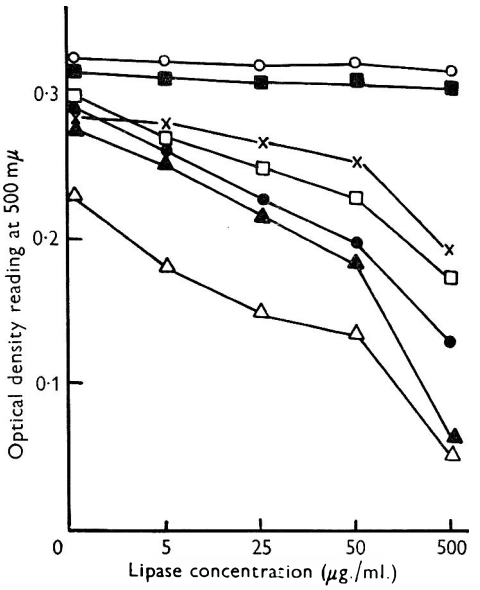

Fig. 11. Lysis by pancreatic lipase. Cells of *Mycoplasma laidlawii* (△), *Mycoplasma mycoides* var. *capri* (▲); L-form of *Streptobacillus moniliformis* (×); spheroplasts of *Escherichia coli* (□); protoplasts of *Micrococcus lysodeikticus* (●); intact cells of *M. lysodeikticus* (○) or *E. coli* (■) were suspended in M-sucrose + 0·067 M-phosphate buffer (pH 7·0) solution containing various concentrations of lipase. Optical density was measured at 37° after incubation for 90 min.

DISCUSSION

The relative resistance of Mycoplasma organisms to osmotic shock has been described by several authors (Smith & Sasaki, 1958; Plackett, 1959; Butler & Knight, 1960). Several hypotheses might be provided to explain the resistance of Mycoplasma to lysis by osmotic shock: (*a*) The very small dimensions and spherical shape of most Mycoplasma organisms decrease the adverse effects of osmotic pressure changes (Mitchell & Moyle, 1956*b*). Thus, the small elements of the L-form of *Proteus vulgaris* were much more resistant to osmotic shock than were the larger elements of this L-form (Thorsson & Weibull, 1958). (*b*) The elasticity of the Mycoplasma envelope might permit considerable distension of the organism without its being ruptured. (*c*) The internal osmotic pressure of the Mycoplasma organisms might be low. There are marked differences between the internal osmotic pressures of different bacteria. Gram-positive cocci, such as *Micrococcus lysodeikticus*, have osmotic pressures of 20–30 atmospheres (Mitchell & Moyle, 1956*b*) while Gram-negative rods have internal osmotic pressure of 4–8 atmospheres only (Gebicki & James, 1960; Hugo & Russell, 1960). Protoplasts of *M. lysodeikticus* therefore underwent almost complete lysis in 0·25 M-sucrose, while spheroplasts of *Escherichia coli* lysed to a similar degree only in 0·03 M-sucrose (Fig. 1). We have no direct way of

measuring the internal osmotic pressure of Mycoplasma cells. However, indirect evidence for a low internal osmotic pressure was obtained while testing the sensitivity of Mycoplasma to lysis by alternate freezing and thawing. Mycoplasma organisms were lysed by this procedure when suspended in de-ionized water, but resisted lysis when sucrose or NaCl were added to the medium (Fig. 3). A concentration of 0·12–0·25 M-sucrose protected almost all Mycoplasma organisms against lysis by freezing and thawing. The protoplasts of *M. lysodeikticus* were lysed by alternate freezing and thawing, even when suspended in M-sucrose solution (Fig. 3). The protective effect of these solutes might be explained by the shrinkage of the plastic Mycoplasma cells by osmotic dehydration in hypertonic media (Postgate & Hunter, 1961). The cell envelope of the shrunken cell is capable of resisting more efficiently the shearing forces of the ice crystals. If this explanation be correct, then 0·25 M-sucrose solution has to be regarded as hypertonic, and 0·12 M-sucrose as about isotonic, to the Mycoplasma organisms. The osmotic pressure of a 0·12 M-sucrose solution is about 3 atmospheres (*Handbook of Chemistry & Physics*, 1954–1955). Hence, the internal osmotic pressure of Mycoplasma organisms seems to be remarkably low.

The marked resistance of the stable L-form of *Streptobacillus moniliformis* to lysis in hypotonic media might be explained along the same lines as the resistance of Mycoplasma organisms to osmotic shock. Medill-Brown, Hutchinson & Cocklin (1960), on comparing the osmotic fragility of penicillin-induced spheroplasts and L-forms derived from *Proteus mirabilis*, found that the spheroplasts were much more sensitive to osmotic shock than were the L-forms. The agglutination of the Mycoplasma organisms by spermine and other polyamines indicates that the cell envelope of Mycoplasma has a net negative charge, like that of the bacterial protoplast membrane (Few, Gilby & Seaman, 1960) and of bacterial cell walls (Razin & Rozansky, 1959). The great sensitivity of Mycoplasma organisms to surface active substances is well known (Klieneberger-Nobel, 1962). Our studies have shown the close similarity between Mycoplasma organisms and bacterial protoplasts with respect to lysis by surface active agents. Mycoplasma organisms were lysed by about the same concentrations of ionic detergents as were bacterial protoplasts. Apparently the ionic detergents act on similar components present in the membranes of Mycoplasma organisms and of bacterial protoplasts, as indicated by the inhibition of lysis when the organisms were pre-treated with uranyl nitrate. Uranyl ions have a strong affinity for the phosphate groups of phospholipids present in the bacterial membrane (Gilby & Few, 1960*a*). Since the cationic detergent cetyltrimethylammonium bromide (CTAB) combines with these acidic groups, its lytic action is inhibited more efficiently by uranyl ions than by the anionic detergent sodium lauryl sulphate (SLS), which combines mainly with the protein component of the membrane (Gilby & Few, 1960*a*). The cationic detergent CTAB causes lysis of Mycoplasma organisms and bacterial protoplasts only within a narrow limit of concentrations. At concentrations higher than 5×10^{-5} M this detergent caused protein denaturation and 'fixation' of the organisms, as well as agglutination by neutralization of surface negative charge (Dawson, Lominski & Stern, 1953).

The similar sensitivity of Mycoplasma organisms and bacterial protoplasts to lysis by primary alcohols and pancreatic lipase suggests the presence of a lipid component in the Mycoplasma cell envelope. Gilby & Few (1960*b*) claimed that

alcohols act on the lipid component of the protoplast membrane, causing lysis by disruption of membrane permeability. The marked lysis by alkali of all the organisms tested, except intact bacteria, is apparently due to the hydration and dispersion of cell proteins (Grula & Hartsell, 1957). The mucopeptide polymer of the bacterial cell wall is alkali resistant and protects the intact bacterial cell from lysis. Digestion of the mucopeptide by lysozyme renders the bacteria sensitive to lysis by alkali (Grula & Hartsell, 1957).

The most remarkable difference between Mycoplasma organisms and all the other organisms examined was the sensitivity of Mycoplasma to lysis by digitonin and saponin; lysis of Mycoplasma by digitonin was reported by Smith & Rothblat (1960). This might be explained by the presence of cholesterol in the Mycoplasma cell envelope (Rothblat & Smith, 1961; Razin & Argaman, unpublished data). The much lower sensitivity of *Mycoplasma laidlawii* to lysis by digitonin when grown in a cholesterol-free medium (Fig. 10) suggests that at least most of the cholesterol in this Mycoplasma species derives from the growth medium.

Our studies show that bacterial L-forms and spheroplasts are less sensitive to lysis than bacterial protoplasts and Mycoplasma organisms. The higher resistance of L-forms and spheroplasts to lysis might be attributed to the presence of a modified cell wall (Thorsson & Weibull, 1958; Klieneberger-Nobel, 1960). The present findings and the morphological studies of ultrathin sections of Mycoplasma organisms (van Iterson & Ruys, 1960) suggest that the Mycoplasma organisms are limited by a membrane of lipoprotein nature, very like that of the plasma membrane which encloses the bacterial protoplast. Chemical analysis of isolated Mycoplasma cell membranes also supports this assumption (Razin & Argaman, to be published). The only difference so far found between the cell membrane of Mycoplasma and the plasma membrane of bacteria is the presence of cholesterol in the former.

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