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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.

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

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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

in English and should in general be divided into the following parts in the order indicated: (a) Summary: brief and self-contained; (b) Introduction; (c) Methods; (d) Results (illustrative protocols only should be included); (e) Discussion (if any), and general conclusions; (f) acknowledgements; (g) References.

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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. 1914a, 1914b, or 1914a, b).

References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper. Titles of journals should be abbreviated in accordance with the *World List of Scientific Periodicals*, 3rd edn. (1952). References to books and monographs should include year of publication, title, edition, town of publication and publisher, in that order. It is the *duty of the author to check his references* and see that the correct abbreviations are used.

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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 there 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 Koibong Li, Barksdale, L. & Garmise, L. (1961).
J. gen. Microbiol. **24**, 355–367.

On p. 358, line 22:

for: 'only Lac$^+$'
read: 'only Lac$^-$'

On p. 363, line 40:

for: 'similar results obtained'
read: 'similar results have been obtained'

On p. 366, line 3:

for: '(T7^{Sh}, 136-R 4)'
read: '(T7^{Sh}, Sh 15)'

In Bungay, C. (1961). *J. gen. Microbiol.* **24**, 393–400.

On p. 394, line 42:

for: '...coverslip and into each circle was pipetted a solution
of 0·01 % (w/v) fibrinogen...'
read: '...coverslip and into each circle was pipetted 0·01 ml. of
a 1·5 % (w/v) solution of fibrinogen...'

The Type Species of the Genus *Nocardia*

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SUMMARY

Expanded descriptions of *Nocardia asteroides* (Eppinger) Blanchard, based on 142 strains, and of *N. brasiliensis* (Lindenberg) Castellani & Chalmers, based on 62 strains, are presented and compared. Illustrations are given of the variation in morphology and appearance of the growth among strains of both species. The morphological and physiological similarity of ATCC strain no. 3318, the accepted type strain of *N. farcinica* Trevisan, to strains of *N. asteroides* is shown, and the resulting problem of nomenclature is discussed.

INTRODUCTION

During the assembly of a collection of strains of mycobacteria, nocardias and streptomycetes, many of the specifically named strains of nocardias received from culture collections and individual investigators were labelled *Nocardia asteroides* (Eppinger) Blanchard. Among these were freshly isolated strains and strains which had been maintained *in vitro* for different periods of time, some for more than 30 years (Gordon & Mihm, 1957). They differed greatly in their macroscopic appearance, formation of pigments, acid-fastness and fragmentation of their vegetative hyphae. Some exhibited rudimentary and very short aerial hyphae; some, abundant aerial hyphae of various lengths; and the aerial hyphae of others segmented into chains of bead-like spores (Gordon & Mihm, 1958). Despite these differences, the strains of *N. asteroides*, new and old, possessed a distinctive group of correlating characteristics. Variants of several strains selected in our laboratory and strains, originally the same, from different donors also had the same distinguishing characters.

Among the other strains in our collection, 26 specifically unidentified isolates from soil or from infections in man or animals, and 23 isolates representing 17 other species, possessed the same group of characteristics identifying *Nocardia asteroides*. The 49 strains were accepted, therefore, as strains of *N. asteroides*. Three of these, two of which were American Type Culture Collection (ATCC) strain no. 3318 obtained from different donors, bore the name *N. farcinica* Trevisan (1889), the name of the type-species of the genus *Nocardia* and a name having priority over *N. asteroides*. In addition, ATCC strain no. 3318 had been accepted as the type strain of the species *N. farcinica* (Waksman, 1957). Although the original description of *N. farcinica* (Nocard, 1888) is meagre, it seemed in our opinion to apply to ATCC strain no. 3318 and to strains of *N. asteroides*. A proposed solution of this problem of nomenclature was postponed, however, until further examination could confirm the similarities or reveal differences between ATCC strain no. 3318 and

Table 1. *Additional strains identified as* Nocardia asteroides *(Eppinger) Blanchard*

Laboratory no.	Name when received, source, and strain name or number
387, 404	*Nocardia asteroides* (Eppinger) Blanchard; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (9907, McCallum; 9935, chicken)
784	*N. asteroides*; B. W. Lacey, Westminster School of Medicine (A_6); NCTC = (British) National Collection of Type Cultures
856, 857	*N. asteroides*; E. Meyer, Univ. of Illinois (Lerner, brain abscess; Suddeth, pulmonary infection)
858–861	*N. asteroides*; W. D. Jones, Jun., Dept. of Public Health, Atlanta, Ga. (1, 2, 3, 4; sputum)
862	*N. asteroides*; W. H. Trejo, E. R. Squibb & Sons, New Brunswick, N.J. (Meade, lesion on knee)
874	*N. asteroides*; I. Uesaka, Kyoto Univ., Kyoto, Japan (Hiroshima, skin ulcer)
886	*N. asteroides*; B. Gueft, Veterans Administration Hospital, Cincinnati, Ohio (sputum)
923	*N. asteroides*; F. B. Seibert, Univ. of Pennsylvania (P.H.S. 2412); N. F. Conant (2412, abscess)
*	*N. asteroides*; E. Wolinsky, Cuyahoga County Hospital, Cleveland, Ohio (Stevens, sputum)
*	*N. asteroides*; S. M. Finegold, Veterans Administration Center, Los Angeles, Calif. (Wherry, empyema)
1011	*N. bostroemi*; N. M. McClung, Univ. of Georgia (159); J. D. Schneidau, Jun. (303); A. Batista (630); Inst. Pasteur (321)
3410	*N. paraffinae* (Jensen) Waksman & Henrici; NCTC (3488)
3423	*N. rangoonensis* (Erikson) Waksman & Henrici; American Type Culture Collection (ATCC); NCTC (1678)
615	*Nocardia* sp.; E. N. Azarowicz, Univ. of Calif. (27)
*	*Nocardia* spp.; M. P. Lechevalier, Rutgers Univ. (1, 10 (7); soil)
*	*Nocardia* spp.; C. I. McDurmont, Dept. of Public Health, Montgomery, Ala. (58–19 467, 58–19 753)
*	*Nocardia* sp.; J. B. Fischer, Dept. of Health, Toronto, Canada (1211)
*	*Nocardia* sp.; E. Wolinsky (Gallagher, sputum)
*	*Streptomyces fradiae* (Waksman & Curtis) Waksman & Henrici; J. K. Johannesson, Wellington City Council, Wellington, New Zealand (A 5, A 22, A 23, A 47; water)
*	*Streptomyces* sp.; A. Grein, Rutgers Univ. (273, water)
1018	*Streptomyces* sp.; J. B. Routien, Chas. Pfizer & Co., Brooklyn, N.Y. (AP 9831, 38540–16; soil)
3305	Unidentified strain; E. Baldacci, Univ. of Milan (22 of series of 25 strains of actinomycetes distributed for study)
933–935, 1023	Unidentified isolations; C. C. Campbell, Walter Reed Army Medical Center (37, 50, 49, pulmonary lesions; 182, ulcerating abscess)
947	Unidentified isolation; H. Keltz, Dept. of Public Health, Columbus, Ga. (P.H.S. 6, sputum of healthy person)
1001	Unidentified isolation; M. L. Littman, Mount Sinai Hospital, New York, N.Y. (MS. 1072)
1024	Unidentified isolation; C. W. Emmons (CM B-404); L. K. Joe (mycetoma)
1826	Unidentified isolation; J. H. Hanks, Leonard Wood Memorial, Culion Palawan, Philippines (blood of patient with Hansen's disease)
3573	Unidentified isolation; R. Holt, Touro Infirmary, New Orleans, La. (breast abscess)
*	Unidentified isolation; J. F. Welsh, Lankenau Hospital, Philadelphia, Pa. (A-59-38, adrenal gland)
*	Unidentified isolation; J. B. Fischer (7-892, thoracic sinus)
*	Unidentified isolation; J. Walker, London School of Hygiene and Tropical Medicine (5859); E. Agius (pulmonary lesion)

* Examined, but not added to this collection.

strains of *N. asteroides,* and until opinions could be obtained from other investigators who had made similar studies.

Strains of *Nocardia asteroides* and *N. brasiliensis* identified since our last report (Gordon & Mihm, 1959) are listed here; the results of further comparative study of the two species are presented; a presumptive test for the recognition of strains of *N. asteroides* is described; and the nomenclature and importance of the type species of the genus *Nocardia* are discussed.

Table 2. *Additional strains identified as* Nocardia brasiliensis
(*Lindenberg*) *Castellani & Chalmers*

Laboratory no.	Name when received, source, and strain name or number
887	*Nocardia brasiliensis* (Lindenberg) Castellani & Chalmers; J. L. Miranda, Inst. Oswaldo Cruz (229)
1093	*N. brasiliensis*; N. F. Conant, Duke Univ. (0 S130); C. M. Whorton (tumour on heel of patient)
1113–1116	*N. brasiliensis*; L. F. Bojalil, Univ. Nacional Autónoma de México (N-37, N-29, N-30, and N-38; mycetomas)
1117A, 1117B	*N. brasiliensis*; L. F. Bojalil (variants of N-114; mycetoma)
1118–1120	*N. brasiliensis*; L. F. Bojalil (N-112, N-113, fistulas; N-115, mycetoma)
1108	*Nocardia* sp.; J. P. Truant, Henry Ford Hospital, Detroit, Mich. (Philip Harris, pustule on forearm)

METHODS

The strains of *Nocardia asteroides* and of *N. brasiliensis* used included those given in Tables 1 and 2 and those listed previously (Gordon & Mihm, 1957, 1959). The observations and tests described by Gordon & Mihm (1957) were used in this study with the following additions.

Survival at 50°. Two slopes of yeast glucose agar were inoculated from a 14- to 30-day culture in glucose broth. One of the two slopes was quickly heated to 50° in a water bath, then held in another water bath at 50° inside a constant temperature incubator for 8 hr. After heating, the slope was quickly cooled, incubated at 28° for 21 days and observed for growth. The unheated culture was also incubated at 28° for 21 days and inspected for growth.

Decomposition of hypoxanthine. Each culture was streaked once across a plate containing *c.* 20 ml. of the following medium: peptone, 5 g.; beef extract, 3 g.; agar, 15 g.; hypoxanthine (Nutritional Biochemicals Corp., Cleveland, Ohio), 5 g.; distilled water, 1000 ml.; pH 7·0. Care in preparation was required to insure an even distribution of the crystals of hypoxanthine throughout the medium. After 14 and 21 days of incubation at 28°, each plate was observed for the disappearance of the crystals of hypoxanthine underneath and around the growth. The importance of a heavy inoculum when testing for the decomposition of casein and of crystals of hypoxanthine, tyrosine or xanthine needs to be stressed. Although some cultures grew well on these media, they did not dissolve the casein or the crystals except around the larger clumps of inoculum.

Sensitivity to lysozyme. Glycerol broth containing 0·005 % (w/v) lysozyme was prepared by dissolving 0·1 g. lysozyme (Nutritional Biochemicals Corp., Cleveland, Ohio) in about 66 ml. sterile 0·01 N-HCl in a 100 ml. volumetric flask plugged with cotton. The solution was boiled over an open flame for 20 min., cooled to room

temperature and brought to 100 ml. by addition of sterile 0·01 N-HCl. Five ml. of the lysozyme solution was mixed with 95 ml. sterile glycerol broth (peptone, 5 g.; beef extract, 3 g.; glycerol, 70 ml.; distilled water, 1000 ml.; pH 7·0) and dispensed in 2·5 ml. amounts in sterile plugged tubes. A loopful of a 14- to 30-day culture in glucose or glycerol broth was inoculated into a tube of lysozyme broth and into a tube of glycerol broth. After 14 and 28 days of incubation at 28°, the tubes were observed and compared for growth.

RESULTS

Nocardia asteroides (*Eppinger*) *Blanchard*

Among the 44 strains of *Nocardia asteroides* listed in Table 1, the specific names *N. bostroemi*, *N. paraffinae*, *N. rangoonensis* and *Streptomyces fradiae* had not been encountered previously among the strains assigned to *N. asteroides*. Although an authority for the transfer of *Actinomyces bostroemi* Baldacci (1937) to the genus *Nocardia* was not found, strain no. 1011 appeared to be in agreement with Puntoni's (1931) description, the description to which Baldacci referred when he proposed the name *A. bostroemi*. Strain no. 3410, received as *N. paraffinae* (Jensen, 1931) Waksman & Henrici (1948), seemed to conform to Jensen's original account. Strain no. 3423 was obtained as *N. rangoonensis* (Erikson, 1935) Waksman & Henrici (1948) from the ATCC more than 20 years ago and corresponded to the first description of the species. Strain no. 6860 received in 1951 from the ATCC as *N. rangoonensis*, however, proved to be unlike strain no. 3423 and to disagree with Erikson's delineation. Strains nos. 1011, 3410 and 3423 were accepted, therefore, as authentic. The four strains of *N. asteroides* received as *S. fradiae* (Waksman & Curtis, 1916) Waksman & Henrici (1948) were very different from authentic strains of *S. fradiae* and were regarded as misnamed.

In 1957 Gordon & Mihm reported the (British) National Collection of Type Cultures (NCTC) strain no. 6531, received as *Nocardia gardneri*, to be one of *N. asteroides* and at variance with its original description (Waksman *et al.* 1942). Later, a subculture of the strain maintained at Oxford University and one from the ATCC were examined and found to agree with the original delineation of *N. gardneri* and to be unlike strains of *N. asteroides*. The first strain labelled *N. gardneri* is regarded, therefore, as a victim of human fallibility.

Microscopic appearance. When stained by the Ziehl-Neelsen method, 27 of the 44 strains listed in Table 1 showed slender branching filaments; 13, rods of various lengths and short filaments; 3, coccoid forms, rods and short filaments; and 1, only short rods. The filaments of about 60 % of the cultures did not fragment during the relatively severe treatment of the Ziehl-Neelsen technique. Eleven of the 44 cultures were not acid-fast; a few rods or portions of a few filaments of 19 cultures retained the carbol fuchsin; and 10–80 % of the rods and filaments of the remaining 14 cultures were acid-fast.

The colonies of about 75 % of the 44 cultures were loosely filamentous with long, branching, intertwining, vegetative (substrate) hyphae (Pl. 1, fig. 1). The remaining cultures formed densely filamentous colonies or both densely and loosely filamentous ones. Fragmentation of the undisturbed vegetative hyphae was observed in only five cultures. All of the 44 cultures formed aerial hyphae which were: in 3 cultures sparse and short; in 4 more abundant, long and straight; in 14 sparse or

abundant, branching, twisted and irregular in length; in 7 short or long and coalesced in tufts; in 15 short or long with many side branches, some of which branched in whorls or clusters. The aerial hyphae of 30 % of the cultures segmented into chains of bead-like spores. Aerial hyphae arising from the substrate hyphae pictured in Pl. 1, fig. 1, are illustrated in Pl. 1, fig. 2. Immediately after the vegetative hyphae in fig. 1 were photographed, only the focus of the microscope was changed (lifted), and the aerial hyphae were photographed for fig. 2.

Macroscopic appearance. The growth of the 44 cultures on yeast glucose agar, after 14 days of incubation at 28°, varied from thin and restricted to abundant and spreading. It was flat, finely or coarsely wrinkled, or warty; in colour beige, yellow, pale orange or pink. A whitish coating of aerial hyphae over the growth of all but four of the cultures was easily seen by the unaided eye. Some of the variations in appearance of the growth occurring among strains of *Norcardia asteroides* in this collection are illustrated in Pl. 3, figs. 5–10 and Pl. 4, figs. 11–16. The diversity among variants of the same strain (Pl. 3, figs. 8–10; Pl. 4, figs. 11–14) is as striking as that among different strains.

Physiological characteristics. The properties of the 44 strains listed in Table 1 were like those of the 98 previously examined strains of *Nocardia asteroides*. Results of the observations of the 142 strains are given in Table 3 and compared with those of *N. brasiliensis*. The following group of reactions was distinctive for strains of *N. asteroides* and separated them from strains of other species of *Nocardia* and those received as streptomycetes, namely: inability to attack casein, hypoxanthine, tyrosine, or xanthine; failure to form acid from adonitol, arabinose, erythritol, inositol, lactose, maltose, mannitol, α-methyl-D-glucoside, raffinose, sorbitol, or xylose; resistance to lysozyme and to 50° for 8 hr.; non-utilization of benzoate. Because of the predominance of negative criteria in this identifying group of reactions, the following characteristics were included: acid production from glucose and glycerol, and utilization of acetate, malate, propionate, pyruvate and succinate as carbon sources. Variation by a strain in any one or two of these 24 reactions was allowed and did not prevent its identification as *N. asteroides*.

Strains nos. 3318 and 3399. The two strains received as *Nocardia farcinica* could not be distinguished from those of *N. asteroides* by any of the observations or tests used in this study. Cultures of both strains formed long branching filaments that did not retain the carbol fuchsin. In their lack of acid-fastness, they resembled 37 % of the other strains assigned to *N. asteroides*. Their colonies were filamentous; fragmentation of the substrate hyphae was not seen. Culture no. 3318 produced sparse, straight, branching aerial hyphae, while those of culture no. 3399 were rudimentary or very short. The aerial hyphae of both cultures could not be observed without the aid of a microscope. After 14 days of incubation at 28°, the growth of both cultures on yeast glucose agar was abundant, spreading, coarsely wrinkled, and pale orange in colour (Pl. 4, fig. 16). Strains nos. 3318 and 3399 possessed all 24 physiological characteristics of the group distinguishing strains of *N. asteroides* (Table 3).

Suggested presumptive test. As the use of so many observations and tests for the identification of a strain is not feasible in a diagnostic laboratory, a presumptive test for strains of *Nocardia asteroides* is offered. In our collection of about 1500 strains of mycobacteria and actinomycetes, 99 % of those which form aerial hyphae

Table 3. *Comparison of physiological characteristics*

Property	*Nocardia asteroides* (% positive strains)	Strain no. 3318	Strain no. 3399	*Nocardia brasiliensis* (% positive strains)
Decomposition of				
Casein	0	−	−	98
Hypoxanthine	4	−	−	94
Tyrosine	2	−	−	100
Xanthine	0	−	−	0
Hydrolysis of starch	56	+	−	55
Acid from				
Adonitol	0	−	−	0
Arabinose	0	−	−	0
Erythritol	8	−	−	0
Galactose	24	−	−	92
Glucose	97	+	+	97
Glycerol	99	+	+	98
Inositol	3	−	−	100
Lactose	0	−	−	0
Maltose	7	−	−	4
Mannitol	1	−	−	94
Mannose	16	−	−	68
α-Methyl-D-glucoside	0	−	−	0
Raffinose	0	−	−	0
Rhamnose	32	−	−	0
Sorbitol	0	−	−	0
Xylose	0	−	−	0
Nitrite from nitrate	88	+	−	90
Growth at				
50°	21	+	+	0
45°	42	+	+	2
40°	88	+	+	60
35°	100	+	+	100
28°	100	+	+	100
10°	17	−	−	41
Survival of 50° for 8 hr.	93	+	+	0
Resistance to lysozyme	99	+	+	100
Utilization of				
Acetate	100	+	+	100
Benzoate	0	−	−	2
Citrate	34	−	−	98
Malate	95	+	+	100
Propionate	100	+	+	100
Pyruvate	99	+	+	100
Succinate	94	+	+	100

and do not decompose casein or crystals of tyrosine or xanthine are strains of *N. asteroides*. The reactions of strains of *N. asteroides* to these three tests are compared in Table 4 with those of the other closely related taxa best represented in our collection. If this collection typifies the strains of aerobic actinomycetes encountered in the diagnostic laboratory, a strain which forms aerial hyphae but does not attack casein or crystals of tyrosine or xanthine can be presumed, with reasonable safety, to be a strain of *N. asteroides*.

Table 4. *Reactions to suggested presumptive test for*
Nocardia asteroides

	Decomposition of		
Taxon	Casein	Tyrosine	Xanthine
Nocardia asteroides	−	−	−
N. brasiliensis	+	+	−
N. madurae	+	+	−
Nocardia sp.	−	−	+
Streptomycetes	+	+	−
	+	+	+
	−	+	+

* As these strains, believed to typify a definite taxon, are rarely encountered, more are sought
for study.

Nocardia brasiliensis (*Lindenberg*) *Castellani & Chalmers*

As the 12 strains listed in Table 2 were labelled *Nocardia brasiliensis* or *Nocardia*
sp. when received, new names and new problems in nomenclature were not
encountered.

Microscopic appearance. Cultures of 11 of the 12 strains grown on glycerol agar
for 5 days at 28° exhibited thin, long or short filaments. The remaining culture was
composed of rods and short thin filaments. Two of the 12 cultures did not retain the
carbol fuchsin; one showed a few partially acid-fast filaments; and 10–75 % of the
filaments of the remaining cultures were acid-fast.

The colonies of the 12 cultures were loosely or densely filamentous or both.
Fragmentation of the vegetative hyphae was not observed. All cultures formed
aerial hyphae which varied from sparse to abundant, short to long, and straight to
gnarled. The aerial hyphae of one culture were long and coalescing; those of three
others coiled at the tips in circles or balls. Segmentation of the aerial hyphae into
bead-like spores was not found.

Macroscopic appearance. The growth of the 12 cultures on yeast glucose agar after
2 weeks of incubation at 28° was beige or pale yellow, flat and spreading or wrinkled
and heaped-up. Seven of the cultures were thickly coated with whitish aerial
hyphae; two were sparsely coated; and three did not have aerial hyphae visible to
the unaided eye. These differences in appearance among strains were also demon-
strable among variants of the same strain (Pl. 5, figs. 17–20).

Physiological characteristics. The reactions of the 12 strains presented in Table 2
resembled those of the 50 strains of *Nocardia brasiliensis* previously described.
Characteristics of the 62 strains are listed in Table 3 and compared with those of
N. asteroides. The following properties were the most reliable for distinguishing
strains of *N. brasiliensis* from those of *N. asteroides*: decomposition of casein, hypo-
xanthine and tyrosine; acid formation from inositol and mannitol; inability to
survive 50° for 8 hr. In addition, the following criteria were useful for the separation
of strains of *N. brasiliensis* from other species of *Nocardia* and those received as
streptomycetes: inability to decompose xanthine: no acid formation from adonitol,
arabinose, erythritol, lactose, maltose, α-methyl-D-glucoside, raffinose, rhamnose,
sorbitol or xylose; resistance to lysozyme; failure to utilize benzoate. Production of
acid from glucose and glycerol and the use of acetate, citrate, malate, propionate,

pyruvate and succinate as sources of carbon were positive characteristics of strains of this species. Variation in any one or two of these 27 characteristics did not hinder the identification of a strain as one of *N. brasiliensis*. We regret that we cannot offer at this time a small group of criteria as a presumptive test for the recognition of strains of this species.

Morphological variation among streptomycetes

The variation in morphology and appearance of the growth exhibited by strains of *Nocardia asteroides* (Pl. 3, figs. 5–10; Pl. 4, figs. 11–16) and *N. brasiliensis* (Pl. 5, figs. 17–20) was also observed among strains deposited in our collection as streptomycetes. Variants resembling those pictured in Pl. 6, figs. 21–24 and belonging to each well represented species of streptomycetes in our collection were received or developed spontaneously or artificially in our laboratory. These strains were soft in texture and did not form aerial hyphae which were visible to the unaided eye. The undisturbed substrate hyphae of their colonies fragmented into rods and short filaments (Pl. 2, figs. 3, 4).

DISCUSSION

One of the guiding principles of this taxonomic study is the firm belief that a strain's specific identity should always be inherent in the strain itself. The identification of a strain should not depend on its source or on characteristics easily lost during cultivation *in vitro*. The delineation of a species necessitates, therefore: (1) a study of newly isolated strains, strains maintained in the test tube for many years and their variants; (2) the selection of a distinctive group of correlating criteria common to all these strains. Only the more stable characteristics which persist after years of cultivation or after exposure to different methods of inducing variation can be used for the recognition of a species. Such a distinguishing group of characteristics was found for *Nocardia asteroides*. Strains nos. 3318 and 3399, which were received as *N. farcinica*, possessed the same characters. Further investigation, which has confirmed the similarity of strains nos. 3318 and 3399 to the older strains of *N. asteroides* in our collection, and discussions with other investigators who have made corresponding studies, have strengthened our belief that *N. farcinica* and *N. asteroides* are two different names for the same species.

The first principle of the *International Code of Nomenclature of Bacteria and Viruses* (1958) is the fixity of names. Although the name *Nocardia farcinica* has priority, replacement of the well-established and widely accepted name *N. asteroides* by one which has nearly disappeared from culture collections and current literature does not, to our minds, serve the principle of the fixity of names. We prefer to leave the onus of such a change to others and to use *N. asteroides* as *nomen conservandum*. Regardless of its nomenclature, the species itself is regarded as the type species of the genus *Nocardia*.

Early in this investigation (Gordon & Smith, 1955) it was believed that the genus *Nocardia* could be separated from the genus *Streptomyces* by a group of physiological reactions. As pointed out, however, the genus *Nocardia* was then represented in our collection by a preponderance of strains of *Nocardia asteroides*. After the examination of more strains of other species of *Nocardia* (*N. brasiliensis*, *N. vaccinii*, *N. coeliaca* and *N. madurae*) it was concluded that the two genera could not be

divided by any of the physiological criteria used in this study. The characteristics of acid-fastness, fragmentation of the vegetative hyphae, abundance of aerial hyphae, and segmentation of the aerial hyphae into chains of spores, currently used for the demarcation of *Nocardia* and *Streptomyces*, are variable for *N. asteroides* and for strains of streptomycetes. We do not understand the current practice in taxonomy of ignoring the strains represented in Pl. 5, fig. 20 and Pl. 6, fig. 22. Such strains are legitimate members of a species, occasionally type strains, and a classification which excludes them is incomplete.

Of the characteristics proposed by others for the division of *Nocardia* and *Streptomyces*, the only one which to our knowledge offers a possible means of separating the two genera is the composition of the cell wall, as reported by Cummins & Harris (1958) and others. Many more strains of all the species concerned need to be examined, of course, before this criterion should be accepted for generic delineation. From our own studies thus far, we are inclined to support the opinions of Bradley & Anderson (1958) and others who have combined the two genera. A firm stand on this question cannot be taken, however, until each species of *Nocardia* and those received as belonging to the *Streptomyces* can be thoroughly studied and their more stable, distinctive characteristics found.

The definition of a species of micro-organisms as a group of freshly isolated strains, strains maintained *in vitro* for different periods of time, and their variants, which have in common a set of correlating characteristics separating them from other groups of strains, emphasizes the inadequacy of the representation of a species by a single strain. The few photographs presented here of variants of the same strain and of different strains of *Nocardia asteroides* illustrate that one strain cannot typify the morphology and macroscopic appearance of the species. In a comparative taxonomic study which includes type species of *Nocardia*, representation of *N. asteroides* by strains which exhibit as many as possible of the known variations in morphology and physiology is strongly recommended.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Substrate hyphae of Walker strain of *Nocardia asteroides* on Bennett's agar at 3 days. × 550.

Fig. 2. Aerial hyphae directly above and formed by substrate hyphae of Fig. 1 on Bennett's agar at 3 days. × 550.

PLATE 2

Fig. 3. Fragmentation of substrate hyphae of strain no. 3699 received as *Streptomyces rimosus* on Bennett's agar at 10 days. × 250.

Fig. 4. Early fragmentation of substrate hypha of strain no. 3699 on Bennett's agar at 10 days. × 1000.

PLATE 3

Figs. 5–7. Colonies of three strains of *Nocardia asteroides* on yeast glucose agar at 10 days. × 1·5.

Figs. 8–10. Colonies of three variants of strain no. 3409 of *N. asteroides* on yeast glucose agar at 10 days. × 1·5.

PLATE 4

Figs. 11, 12. Colonies of two variants of strain no. 443 of *Nocardia asteroides* on yeast glucose agar at 10 days. × 1·5.

Figs. 13, 14. Colonies of two variants of strain no. 3045 of *N. asteroides* on yeast glucose agar at 10 days. × 1·5.

Figs. 15, 16. Colonies of two strains of *N. asteroides* on yeast glucose agar at 10 days. × 1·5.

PLATE 5

Figs. 17, 18. Colonies of two variants of strain no. 1117 of *Nocardia brasiliensis* on yeast glucose agar at 10 days. × 1·5.

Figs. 19, 20. Colonies of two variants of strain no. 774 of *N. brasiliensis* on yeast glucose agar at 10 days. × 1·5.

PLATE 6

Figs. 21, 22. Colonies of two variants of strain no. 3535 received as *Streptomyces fradiae* on yeast glucose agar at 10 days. × 1·5.

Figs. 23, 24. Colonies of two variants of NRRL strain no. 2234 received as *S. rimosus* on yeast glucose agar at 10 days. × 1·5.

Fig. 1

Fig. 2

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Fig. 3

Fig. 4

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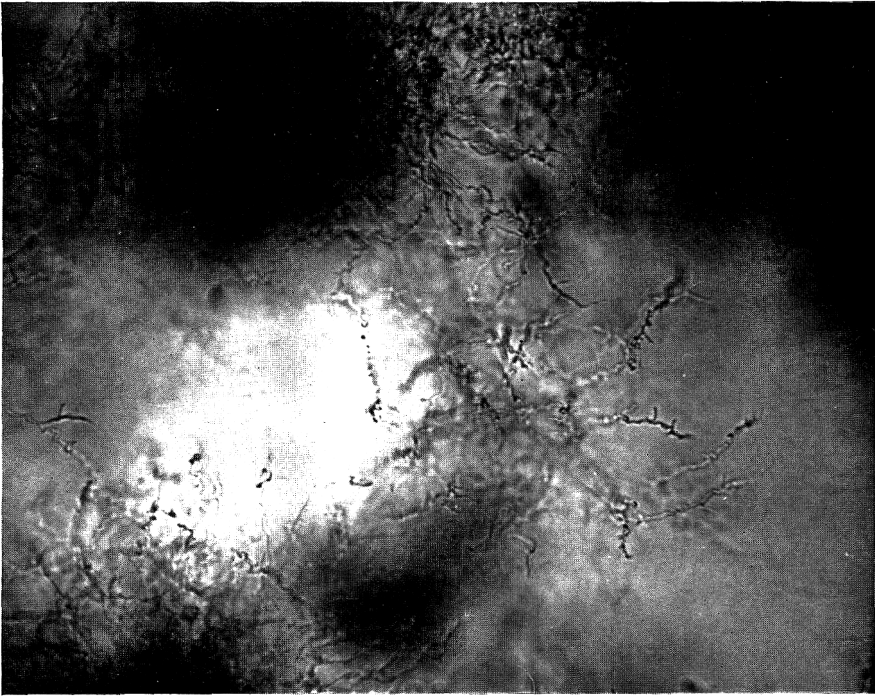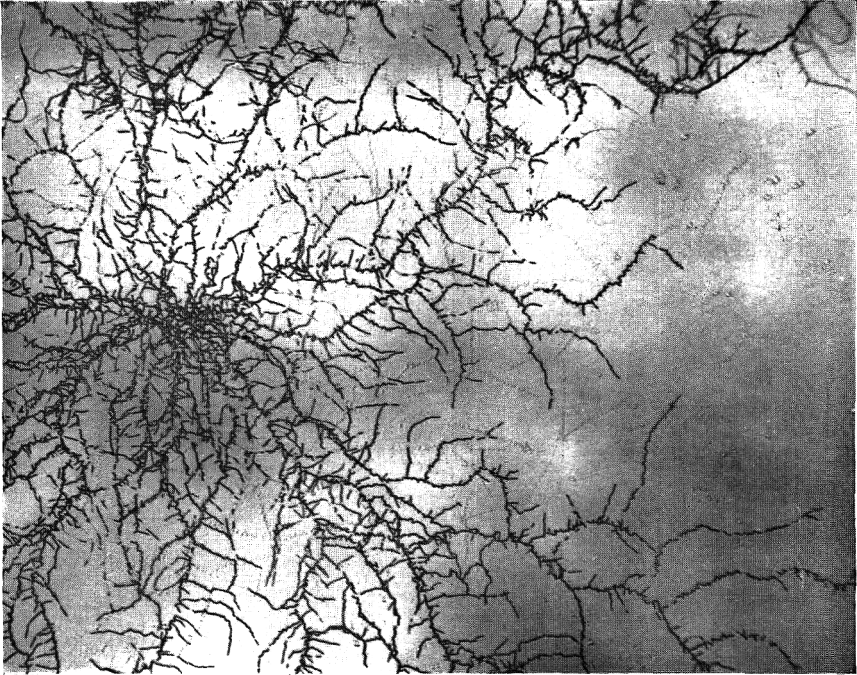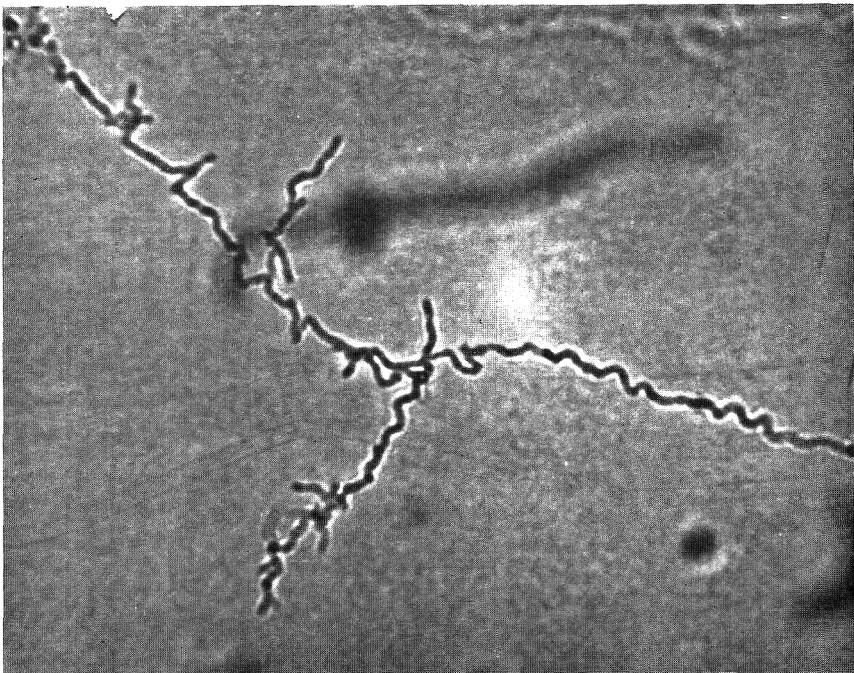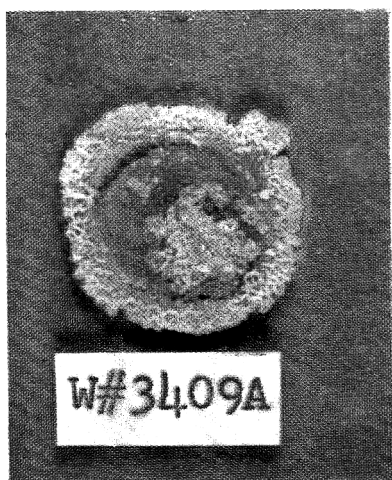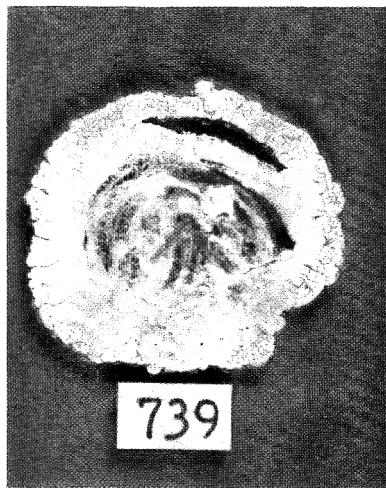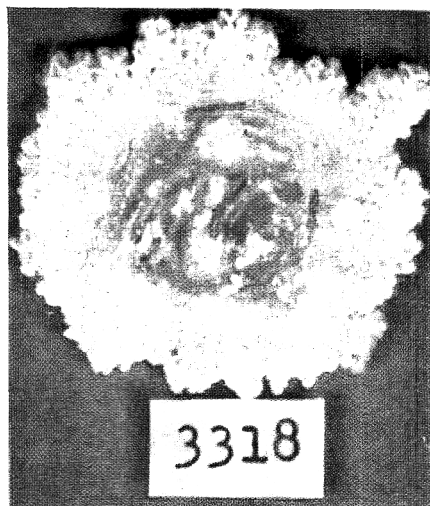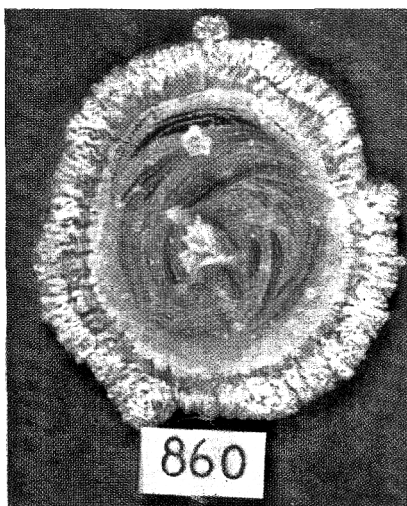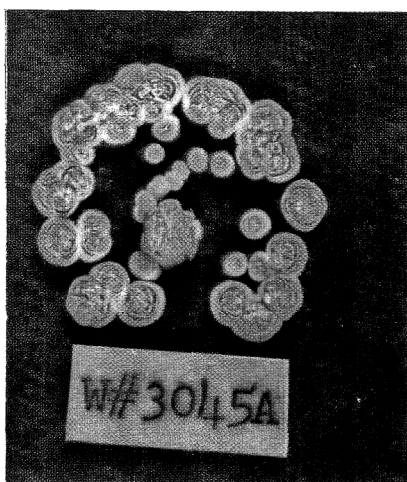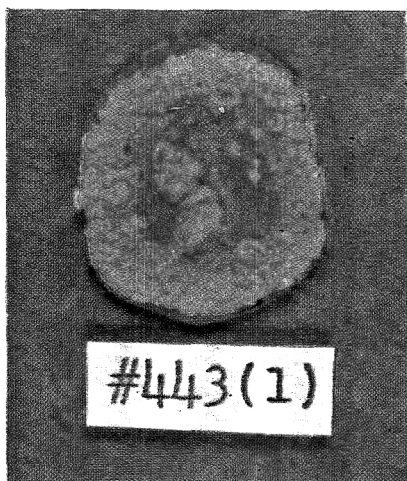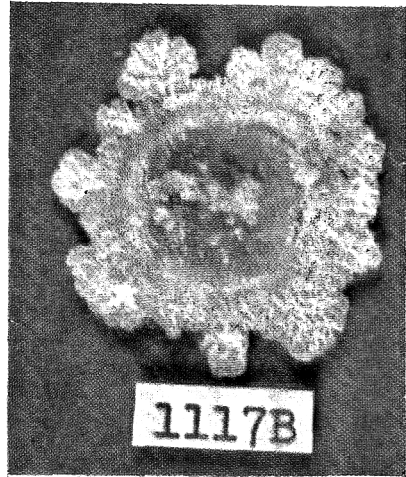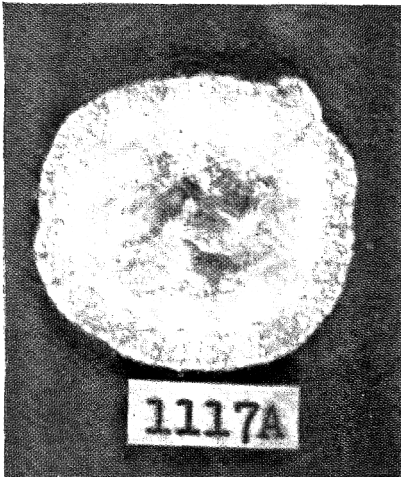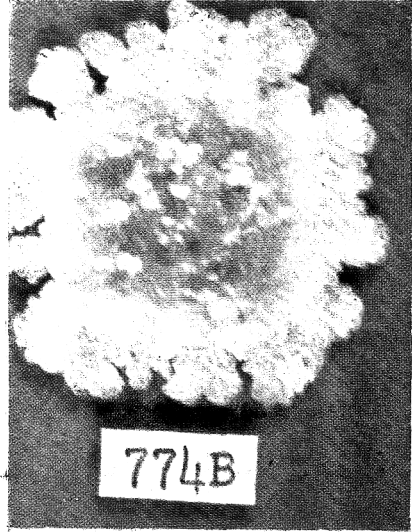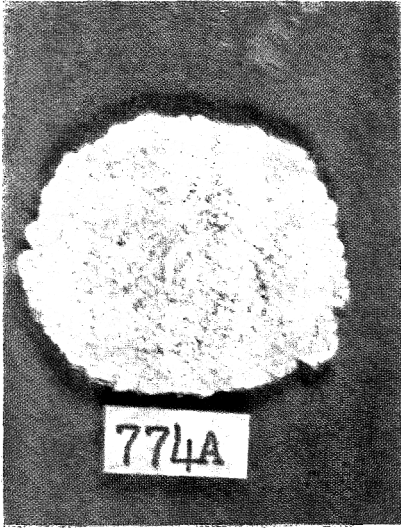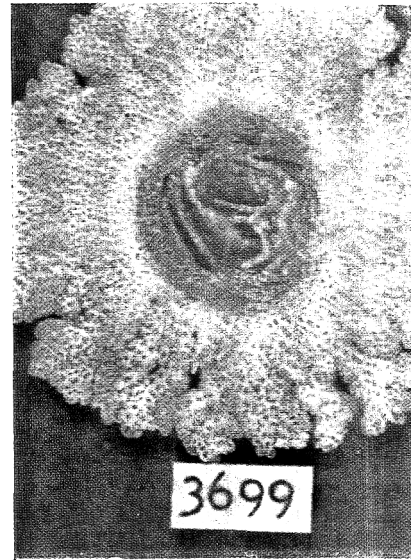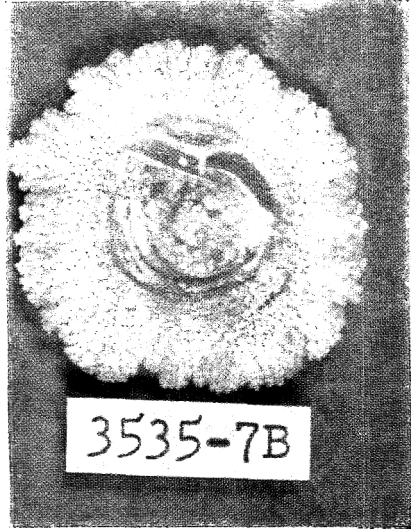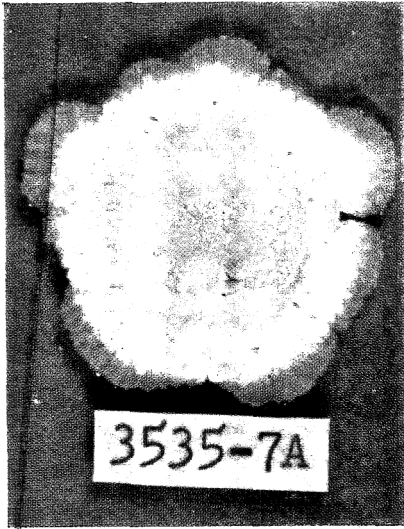
Biological Assay of Cephalosporin C

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SUMMARY

A method of assaying the antibiotic cephalosporin C in low concentration in culture fluids by using a strain of *Vibrio cholerae* is described. The formation of spheroplasts of the test organism in subinhibitory concentrations of the antibiotic and chemically related compounds is discussed.

INTRODUCTION

The antibiotic cephalosporin C was first discovered in crude preparations of cephalosporin N (Newton & Abraham, 1954) and it was found that both substances were produced by the same species of *Cephalosporium* (C.M.I. 49137) in fermentations. Cephalosporin N was then being assayed by a plate diffusion method against a strain of *Klebsiella pneumoniae* by using the technique described by Brownlee *et al.* (1948). Cephalosporin C in eluates from carbon adsorption columns was assayed against the same organism, with cephalosporin N as standard, but this test was not sensitive enough to detect the very low concentrations of cephalosporin C present in culture fluids. Therefore an attempt was made to develop a more sensitive assay for cephalosporin C. About 100 strains of Gram-negative organisms were examined by a gutter plate technique to find an organism more sensitive to cephalosporin C than the *K. pneumoniae*. Gram-positive organisms were not considered since these were likely to be sensitive to cephalosporin P, which was also produced by the Cephalosporium strain used. A strain of *Vibrio cholerae* 1077 proved to be the most sensitive of the organisms tested. This organism had been obtained from Dr I. N. Asheshov, who had used it as a host organism in the assay of the antiphage activity of various substances (Asheshov, Strelitz & Hall, 1949; Hall, Kavanagh & Asheshov, 1951). It had been maintained in the laboratory for many years and was known to be of attenuated pathogenicity. By dilution assays it was shown to be at least ten times as sensitive to cephalosporin C as *K. pneumoniae* and *Salmonella typhi*.

The usual technique for diffusion assay could not be used since *Vibrio cholerae* 1077 did not grow when deep-seeded in agar medium. By using a surface inoculum there was some growth which was considerably improved by using only 0·75 % (w/v) agar in the medium, which was a papain digest medium as used by Dr Asheshov for growing this organism. Since the conditions of the assay are critical, they are described in detail.

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METHODS

Papain digest agar. The original method of preparation of papain digest medium (Asheshov, 1941) was modified. Meat meal (No. 1 Meat Protein, K 24, Meat and Bone Meal; British Glues and Chemicals Ltd., Imperial House, Kingsway, London, W.C. 2) was used instead of fresh meat, and this was digested with a commercial preparation of papain (British Drug Houses Ltd., Poole, Dorset) at 60° for 3 hr. The following quantities were used: meat meal, 400 g.; papain, 24 g.; glycerol, 20 ml.; distilled water, 8 l.

The pH value was kept at 7·0 by adding 10 % (w/v) ammonia. This concentrated digest was cleared by filtration through kieselguhr on paper, autoclaved at 121° for 20 min. and stored until used to make up the final medium which contained concentrated papain digest, 400 ml.; Bacto yeast extract, 2·5 g.; NaCl, 5·0 g.; trace elements solution, 1·0 ml.; distilled water, 600 ml.; adjusted to pH 7·0 with ammonia; agar, 7·5 g. This was sterilized by autoclaving at 121° for 20 min.

The trace element solution contained: H_3BO_3, 0·057 g.; $CuSO_4.5H_2O$, 0·157 g.; $(NH_4)_2SO_4.FeSO_4.6H_2O$, 1·404 g.; $MnSO_4.4H_2O$, 0·081 g.; $ZnSO_4.7H_2O$, 0·079 g.; distilled water to 1000 ml.

Nutrient agar. Oxoid bacteriological peptone, 5 g.; Lab-Lemco, 3 g.; New Zealand agar, 20 g.; distilled water, 1000 ml.; pH 7·4.

Maintenance of test organism. Vibrio cholerae 1077 was grown aerobically on papain digest agar in Petri dishes incubated overnight at 37° and then kept at room temperature. Fresh cultures were put up weekly since the organism died out fairly rapidly, especially at 4°. No loss in sensitivity was observed during the course of this study.

Inoculum. A 4–6 hr. culture in papain digest broth was used to inoculate the assay plates. Papain digest broth (100 ml.) in a 250 ml. conical flask was inoculated by a 24 s.w.g. wire loop 2 mm. in diameter filled with growth from a 24-hr. culture on solid medium, and incubated at 37° for at least 4 hr. without shaking.

Assay plates. Each plate consisted of an aluminium frame 32 cm. square and 3 cm. deep, sealed on to a glass sheet with a layer of nutrient agar (110 ml.). A second layer of papain digest agar (110 ml.) was poured on top of this and the plate left to dry at 32° for 1–2 hr. The lid was a second sheet of glass, covered with filter paper, to absorb condensation moisture.

Inoculation. Enough of the 4-hr. Vibrio cholerae broth culture was poured on to the plate to flood the surface completely, then the excess liquid was pipetted off. This method gave a more even layer of growth over the whole surface than did the addition of a measured amount of broth, just enough to cover the agar. The inoculated plate was allowed to dry at 32° for a further hour, the samples then added at room temperature, and the plate incubated at 32°–37° for 16–20 hr. Since the medium contained only 0·75 % (w/v) agar, holes could not be cut in it, and the samples were placed on the plates by means of Whatman Antibiotic Assay disks, 13 mm. diam., which were dipped in the sample with fine forceps, drained by touching momentarily the rim of the sample container, and laid on the surface of the plate. By this technique each disk soaked up about 0·14 ml. of solution. Plates were incubated at 32° immediately after application of antibiotic.

Standard solutions. When it became available a crystalline preparation of the

hydrated sodium salt of cephalosporin C was used as standard. Solutions of this were made up weekly at 0·4 u./ml. in 0·05 M-phosphate buffer (pH 7·0) and kept at 4°. The concentrations of standard used on the plate were 0·4 and 0·1 u./ml. The cephalosporin C unit was originally based on the cephalosporin N unit assayed against *Salmonella typhi* but was later defined as the activity contained in 0·13 mg. of a Master Standard prepared at Clevedon in June 1958.

Removal of cephalosporin N from samples. Under the conditions at present used for cephalosporin C production, some cephalosporin N was always formed and since *Vibrio cholerae* 1077 was sensitive to cephalosporin N, this had to be removed from culture fluid samples before they could be assayed for cephalosporin C activity. The method adopted depended on the destruction of cephalosporin N by dilute acid in which the cephalosporin C was relatively stable. The conditions were chosen so that the cephalosporin C itself was not inactivated and the formation of cephalosporin N penillic acid was kept as low as possible, since this had some slight activity against *V. cholerae* (G. A. Miller & R. W. Brimblecombe; unpublished observations). Each sample of culture fluid was acidified to pH 2–3 with 2 M-phosphoric acid using a glass electrode, and held at 37° in a water bath for 2 hr. The pH value was then measured, the solution readjusted to pH 7 with N-sodium hydroxide, and the sample incubated at 37° for a further hour to destroy cephalosporin N penillic acid. It was essential that the pH value during this second incubation should not be above 7·5 or the cephalosporin C itself would be destroyed.

Calculation of potency of unknown samples. The arrangement of the samples on the assay plates and the method of calculation was taken from the scheme described in detail by Brownlee *et al.* (1948).

<center>RESULTS</center>

<center>*The assay*</center>

Inhibition zones. The zones formed by cephalosporin C on *Vibrio cholerae* 1077 plates were unusual. There was a zone of complete inhibition of growth, and outside this a zone of abnormal mucoid growth, more opaque than the normal growth on the rest of the plate. The zones were magnified (× 4·5) and projected on to a flashed white opal glass screen to be measured. On this screen the zone of inhibition was white and the zone of abnormal growth dark brown. By measuring the diameter of this outer zone, rather than the zone of actual inhibition, it was possible to increase the sensitivity of the assay considerably. Concentrations of cephalosporin C down to 0·015 u./ml. then gave measurable zones.

Morphology of organisms in the outer zone. Microscopical examination of wet preparations of organisms, suspended in papain digest broth, from a typical zone of abnormal growth showed that the normal comma form of the vibrio had disappeared and the growth consisted of spherical forms. These spheroplasts appeared to consist of an outer membrane within which was dense granular material and frequently large clear vacuoles, often crescent shaped. Similar spherical forms produced by *Escherichia coli, Proteus vulgaris, Aerobacter aerogenes,* and other Gram-negative bacteria in the presence of penicillin have been described by McQuillen (1958, 1960), Lederberg (1956), Hahn & Ciak (1957), Gebicki & James (1960) and others, and in the presence of 6-aminopenicillanic acid by Hugo & Russell (1960). Normal organisms of the strain we used of *Vibrio cholerae* varied in size

from 1·5 to 4·0 μ long × 0·2 to 0·4 μ wide. The spheroplasts varied in diameter from 3·0 to 7·0 μ with occasional large forms up to 10–11 μ in diameter. They were always perfectly spherical in shape. The proportion of spheroplasts to normal bacteria decreased towards the outer edge of the zone.

When suspended in a 10–20 % (w/v) sucrose solution, the spheroplasts remained intact for many hours, but when suspended in distilled water they lysed rapidly, leaving much less optically dense ghost forms and amorphous masses of debris. The spheroplasts were shown by microscopical examination to be formed in the outer zones as soon as growth on the assay plate was visible to the naked eye, i.e.

Fig. 1

Fig. 2
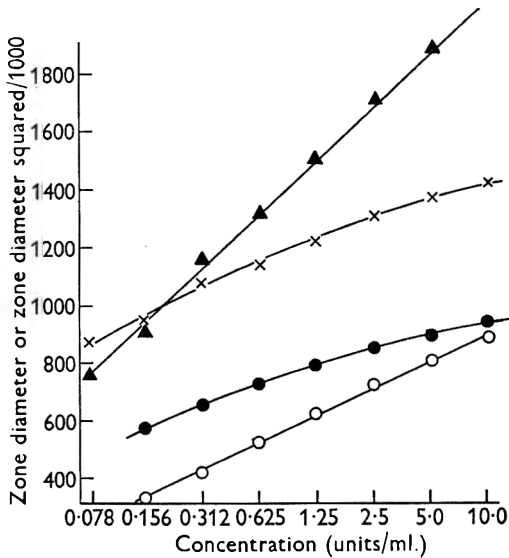
Fig. 1. Zone diameter/log concentration relationship on *Vibrio cholerae* assay plates, of the inner and outer zones of cephalosporin C. All zone diameters as measured on projector in mm. (i.e. × 4·5 magnification), totals of eight zones. ▲——▲, outer zone diameter squared/1000; ×——×, outer zone diameter. ○——○, inner zone diameter squared/1000; ●——●, inner zone diameter.
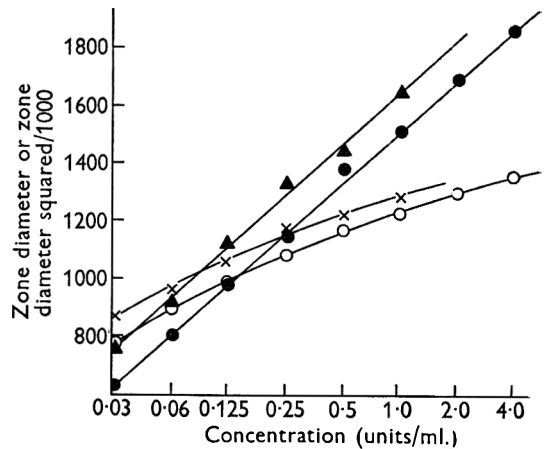
Fig. 2. Outer zone diameter/log concentration curves from *Vibrio cholera* plates. All zone diameters are measured on projector in mm. (i.e. × 4·5 magn.), totals of eight zones. Cephalosporin C: ○——○, zone diameter; ●——●, zone diameter squared/1000. Fermentation broth: ×——×, zone diameter; ▲——▲, zone diameter squared/1000.

after about 3–4 hr. at incubation temperature. When there was no growth of the organism, e.g at 4°, no spheroplasts were formed. When subcultured on to fresh medium, spheroplasts from assay plates produced colonies of normal *Vibrio cholera* organisms.

Outer and inner zones. On certain plates, both the edge of the outer zone and the edge of the inner zone of complete inhibition were measured and from this the width of the zone of spheroplasts determined. The width of this zone, in relation to the total zone diameter, increased as the concentration of the cephalosporin C decreased. Thus at 5 u./ml. cephalosporin C, the width of the spheroplasts zone was 17·3 % of the total zone diameter, but at 0·156 u./ml. it was 20 %. The plot of log con-

centration against the square of the zone diameter for both types of zone was linear, but the two lines were not parallel, as shown in Fig. 1.

Dose response curve. When the assay technique was well established and a solid standard became available, accurate assays were carried out with cephalosporin C standard solutions at eight concentrations from 4·0 to 0·03 u./ml. (eight replicates of each concentration were set up on eight assay plates). The relationship between log concentration and the square of the zone diameter was linear, whereas the plot of log concentration against the zone diameter showed second degree curvature, as is shown in Fig. 2. Culture fluid, from which cephalosporin N had been removed, and which had been shown by chromatography and electrophoresis to contain no acid-stable antibiotic other than cephalosporin C, was tested at several concentrations and the same linear relationship was found between the square of the zone diameter and log concentration (see Fig. 2).

Reproducibility of the assay. Under routine working conditions three samples of cephalosporin C (0·8, 0·4 and 0·1 u./ml.) were assayed against cephalosporin C standard by the normal technique. Twelve replicate plates were filled on 1 day, three by each of four operators. As there was some evidence that this assay showed day-to-day variation, the experiment was repeated, again with twelve plates, one operator filling three plates/day for 4 consecutive days, the samples being kept at 4°. The results are shown in Table 1.

Table 1. *Reproducibility of the cephalosporin C assay for plates filled on one day by four operators and for plates filled on four consecutive days by one operator*

Experiment		Known potency of cephalosporin C sample		
		0·8 u./ml.	0·4 u./ml.	0·1 u./ml.
Twelve plates filled on 1 day by four operators	Estimated potency (mean of twelve results)	0·74	0·40	0·10
	Standard deviation	0·0415	0·0247	0·0095
	Coefficient of variation	5·6 %	6·2 %	9·5 %
Three plates filled on 4 consecutive days— one operator	Estimated potency (mean of twelve results)	0·81	0·40	0·10
	Standard deviation	0·0742	0·0183	0·0080
	Coefficient of variation	9·2 %	4·6 %	7·9 %

Activity of some related antibiotics against Vibrio cholerae

Cephalosporin P at concentrations considerably higher than those produced in either cephalosporin C fermentation or the special cephalosporin P fermentation (Crawford *et al.* 1952) were completely inactive against *Vibrio cholerae*. Cephalosporin N was active on *V. cholerae* plates, down to 1·0 u./ml. and formed zones identical in appearance, macroscopically and microscopically, to cephalosporin C zones.

Penicillin (above 5 u./ml.) formed zones on *Vibrio cholerae* plates similar to those formed by cephalosporin C, but the zone of abnormal growth was narrower as compared with the total zone diameter. For example, penicillin at 125 u./ml. formed about the same size zone as 0·4 u./ml. cephalosporin C; with penicillin the
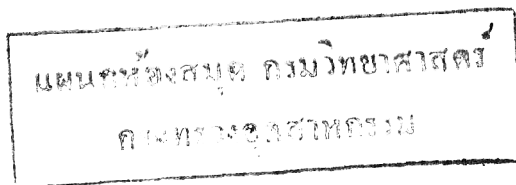
width of the zone of abnormal growth was 8·4 % of the total, whereas with cephalosporin C the width of the 'spheroplast' zone was 18·1 % of the total zone diameter. However, the abnormal growth of the penicillin zone consisted of spheroplasts identical in appearance with those formed by cephalosporins C and N. 6-Aminopenicillanic acid at concentrations above 0·4 mg./ml. gave inhibition zones of the cephalosporin C type with outer zones containing typical spheroplasts, while 0·2 mg./ml. gave only spheroplast zones. With 6-aminopenicillanic acid, the width of the spheroplast zone varied from 13·3 % of the total zone diameter at 3·2 mg./ml.
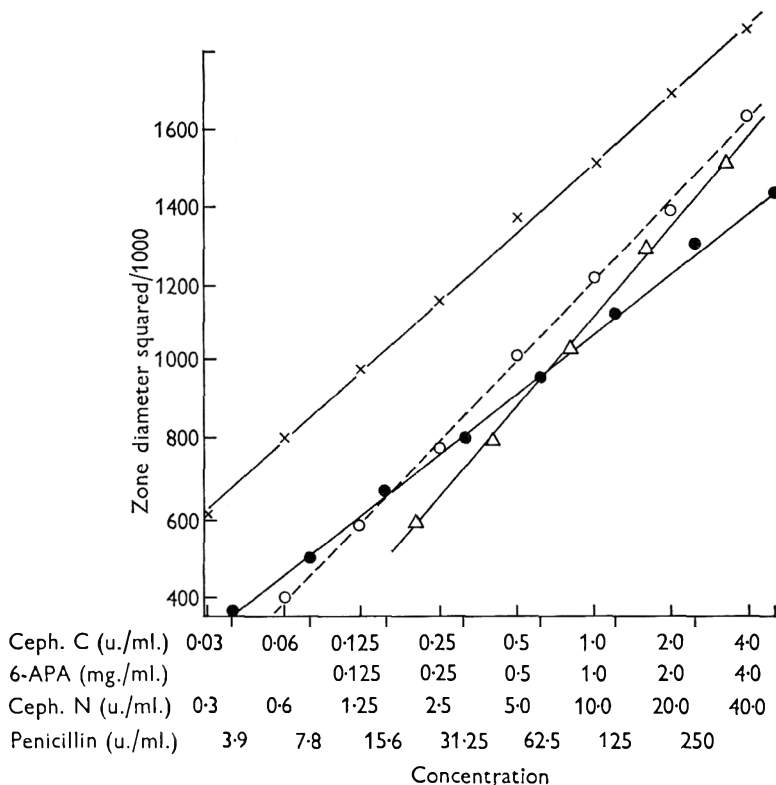
Fig. 3. Outer zone diameter squared/1000/log concentration relationship on *Vibrio cholerae* plates for cephalosporin C, × —— × ; cephalosporin N, ○——○; and penicillin, ●--●; 6-aminopenicillanic acid, △——△. All zone diameters as measured on projector in mm. (i.e. × 4·5 magnification), totals of eight zones.

to 16·1 % at 0·4 mg./ml. When assayed against cephalosporin C standard, 6-aminopenicillanic acid at 3·2 mg./ml. is equivalent to cephalosporin C 0·48 u./ml. The relationships of the square of the zone diameter to log concentration of cephalosporin N, penicillin and 6-aminopenicillanic acid against *V. cholerae* are compared with that of cephalosporin C in Fig. 3.

The quaternary pyridine compound of cephalosporin C, cephalosporin C_A (Hale, Abraham & Newton, 1958, 1961), produced zones on *Vibrio cholerae* plates identical with cephalosporin C zones, although the compound was relatively inactive against *V. cholerae*.

An attempt was made to compare the activity of cephalosporin C and related compounds against *Vibrio cholerae*, *Staphylococcus aureus* H and *Salmonella typhi*. As these compounds are similar in structure and molecular weight, their diffusion constants will be similar. By testing all the compounds on a single assay plate for each organism, measurement of the zones of inhibition gives a valid comparison of their relative activities. Average results from a number of plates of this type are given in Table 2.

Table 2. *Activity of cephalosporin C and related antibiotics*

Test organism	Antibiotic	μg./ml.	Average zone diameters (mm.)
Vibrio cholerae 1077	Cephalosporin C	44·7	28·6
(outer zone of abnormal growth)	Cephalosporin N*	621·0	28·4
measured)	Penicillin	60·0	26·3
	Cephalosporin P	588·0	0
	Cephalosporin C_A (pyridine)	275·0	25·5
Salmonella typhi (Oxford)	Cephalosporin C	1117·3	19·5
(zone of inhibition)	Penicillin	6·0	14·0
	Cephalosporin P	588·0	0
	Cephalosporin C_A (pyridine)	343·0	19·3
Staphylococcus aureus H 6571	Cephalosporin C	1117·3	20·4
(zone of inhibition)	Penicillin	0·3	17·1
	Cephalosporin P	58·8	20·1
	Cephalosporin C_A (pyridine)	137·6	20·3

* Impure sample.

A number of other antibiotics were found to be active against *Vibrio cholerae* but did not produce the cephalosporin C type of zone. Streptomycin, aureomycin, terramycin, chloramphenicol and erythromycin all formed zones of complete inhibition around which there was no abnormal growth, and no spheroplasts were found.

DISCUSSION

The *Vibrio cholerae* assay for cephalosporin C proved extremely useful when only very low concentrations of cephalosporin C were being produced in the culture fluids. Previously, culture fluid samples had to be concentrated by freeze-drying and reconstituted in a small volume after the destruction of cephalosporin N, then assayed against *Klebsiella pneumoniae*. With the *V. cholerae* assay, culture fluids could be assayed without concentration; after the destruction of cephalosporin N, any cephalosporin P present could be ignored. Removal of cephalosporin N by acid inactivation was essential in samples from these early fermentations, where the amount of cephalosporin C was very low as compared with cephalosporin N. As fermentation conditions were improved and the yield of cephalosporin C considerably increased, this step was not so necessary, since the amount of cephalosporin N present was not enough to affect significantly the higher cephalosporin C titres.

However, any change in fermentation conditions was likely to alter the balance between cephalosporin C and cephalosporin N, and a frequent check on this would be needed if the cephalosporin N were not routinely destroyed before assay of cephalosporin C.

Newton & Abraham (1956) stated that cephalosporin C was insensitive to some preparations of penicillinase, and therefore the destruction of cephalosporin N by incorporation of penicillinase in the assay medium was possible. However, this technique cannot be recommended until more is known of the relative stability of cephalosporin C to different penicillinase preparations under varying conditions.

The best linear fit for log dose response curves was obtained with the square of the zone diameter, when a large number of replicate zones were measured. It has been shown (Gridgeman, 1943; Wood, 1944) that where a four-point assay technique is employed, second-degree curvature does not affect the estimate of potency ratio. In most instances, the approximate titre of the samples to be assayed was known, and they could be diluted to within the range of the standards. Humphrey & Lightbown (1952) showed that under these conditions the use of the square of the zone diameter in calculation was justified only if very accurate assays were required.

The action of cephalosporin C against *Vibrio cholerae* was similar to that of 6-aminopenicillanic acid and penicillin, apparently affecting cell wall synthesis and causing the formation of spherical bodies. The osmotic sensitivity of these spherical forms and their ability to develop into normal bacillary organisms showed that these were spheroplasts with an incomplete cell wall, but not true protoplasts devoid of all cell wall material, as described by Brenner *et al.* (1958). In view of the similarities which exist between the chemical structures of cephalosporin N (Newton & Abraham, 1954), cephalosporin C (Abraham & Newton, 1961), the cephalosporin C_A derivatives (Hale, Abraham & Newton, 1961), and penicillin, and of the common relationship to 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959), it was of interest to find that these compounds all show spheroplast-forming action similar to that of 6-aminopenicillanic acid against this strain of *V. cholerae*. No abnormal growth forms other than spheroplasts were found in these zones, at any concentration of these antibiotics. Cephalosporins C and N caused the formation of spheroplasts over a wider range of concentrations than penicillin and therefore formed larger zones of abnormal growth on *V. cholerae* assay plates.

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Decomposition of Lignin by Soil Bacteria and Complex Formation between Autoxidized Lignin and Organic Nitrogen Compounds

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SUMMARY

Several bacteria able to decompose native lignin prepared according to Brauns (1939) were isolated from soil; they were aerobic, Gram-negative, motile or non-motile, non-endospore forming rods belonging to the genera *Pseudomonas* and *Flavobacterium*. The decomposition was studied in a neutral medium containing the lignin as a suspension of fine particles and solidified with silica gel. 20–30 % of the lignin added to the medium was decomposed by the bacteria. Independently of the biological decomposition, a non-biological transformation of lignin occurred during the later stage of the incubation when the medium dried out. The products of the non-biological transformation were dark brown, soluble in water but insoluble in ethanol. They reacted readily with nitrogen compounds in peptone, forming complexes containing about 2·5 % nitrogen, half of which was α-amino nitrogen. The relation of these complexes to soil humic acid is discussed.

INTRODUCTION

Lignin, cellulose and xylan are the major constituents of the plant residues being decomposed in the soil. Cellulose and xylan are known to be decomposed by a large number of aerobic and anaerobic micro-organisms by the action of hydrolytic enzymes. Lignin disappears from decomposing plant material, but slowly as compared with polysaccharides, and little is known about the organisms responsible for this decomposition. Certain fungi mostly belonging to the Basidiomycetes (*Polyporus* spp., *Marasmius* spp.) are known to be able to utilize lignin from straw and wood; these organisms, however, utilize the cellulose and xylan constituents as well. A few species appear to prefer the lignin to the polysaccharides (Lindeberg, 1946).

The sparse information available about this important process is partly due to the difficulties involved in preparing pure unaltered lignin. The preparations mostly used have been isolated by means which make it doubtful whether the material obtained represents unaltered lignin. The method introduced by Brauns (1939) which uses only neutral solvents was a great improvement in this respect. Lignin prepared according to this method was first used by Day, Pelczar & Gottlieb (1949) for investigating the microbial decomposition. Reviews of the literature dealing with the methods of preparation and biological decomposition of lignin have been presented by Norman (1937), Gottlieb & Pelczar (1951) and Lawson & Still (1957).

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ห้องสมุด กรมวิทยาศาสตร์

Recent literature on lignin and formation of soil organic matter was reviewed by Bremner (1954). The present paper describes the isolation of lignin-decomposing bacteria from soil, the quantitative determination of the decomposition and the chemical analyses of the lignin and its decomposition products.

METHODS

Media

Culture media. The basal mineral solution had the following composition (%, w/v, dissolved in tap water): K_2HPO_4, 0·05; $MgSO_4 7H_2O$, 0·02; NaCl, 0·02; $FeSO_4$, 0·01. Medium GN contained in addition to the basal medium 0·1% (w/v) glucose and 0·05% (w/v) NH_4NO_3; medium GP 0·5% (w/v) glucose, 0·1% (w/v) Bacto peptone (Difco), and 0·05% yeast extract. Agar to 1·8% (w/v) was added when the media had to be solidified. Soil extract (25%, v/v) was added to the media in some cases; this extract was prepared by autoclaving 1 kg. soil suspended in 1 l. water for 30 min. at 110°, followed by filtration.

The lignin preparation. Wood flour (a mixture of pine and spruce of commercial Norwegian origin) was extracted with water at room temperature until the extract was colourless. The wood flour was dried and then extracted with 96% (v/v) ethanol in water for about a week, i.e. as long as lignin was extracted. The ethanol extract was evaporated to dryness under reduced pressure and the residue treated with ether and water until it became powdery, then purified by reprecipitations until the methoxyl (—OCH_3) content was constant as described by Brauns (1939). About 3 g. purified material was obtained from 1 kg. wood flour. The preparation was a light cream-coloured powder soluble in N-sodium hydroxide, dioxane, ethanol, methanol, pyridine and insoluble in water, ether, benzene, light petroleum. It gave the typical purple colour reaction with phloroglucinol + hydrochloric acid. Chemical analysis: 61·24% C, 6·33% H (determined by the Chemical Department, Danish Atomic Energy Commission Research Establishment Risö), 0·2% ash, 86·0% Klason lignin, 12·9% —OCH_3, 0·17% N, 0·03% α-amino acid nitrogen. Paper chromatograms revealed the presence of amino acids and sugars in hydrolysates (see later). Brauns (1939) gave the following analytical data for native lignin from spruce wood: 63·89% C, 6·07% H, 14·9% —OCH_3.

Preparation of sterile suspension of lignin in water. A homogenizer made from two close fitting Pyrex test tubes (see Umbreit, Burris & Stauffer, 1949, p. 136) was sterilized in the oven. A portion of lignin was brought into the tube of the homogenizer and absolute ether added until the lignin was covered by a layer 10 cm. deep; the tube was then closed with a sterilized cork stopper and left for 2–3 days. Most of the ether was removed by decantation and the cork stopper replaced by a sterilized cotton plug. When all the ether had evaporated through the cotton plug sterile water was added and a homogeneous suspension prepared by means of the pestle of the homogenizer, moved by the hand. The suspension was brought aseptically into a sterile flask, adding sterile water to make a 2% (w/v) suspension. The exact concentration was determined by a dry wt. determination on a sample. The sterility of the suspension was tested by inoculation of nutrient agar and nutrient broth with a drop of the suspension. Microbial growth was never observed from a lignin suspension prepared in this way.

Silica gel + lignin medium. An aqueous solution which contained 1·8–2·0 % (w/v) sodium silicate was prepared from sodium water-glass. The solution was run through a column of Amberlite IR 120 (H) (Taylor, 1950; Smith, 1951) and autoclaved at 110° for 20 min. The solution after autoclaving was at pH 3·0. Two mineral solutions were prepared: solution (A) contained (%, w/v): NaCl, 1·0; NH_4NO_3 or KNO_3, 0·6; K_2HPO_4, 0·3, $MgSO_47H_2O$, 0·1; $FeSO_4$, 0·05; solution (B) contained the same constituents but in double strength. The salts were dissolved in tap water and sterilized by autoclaving. A few ml. of sterile N-sodium hydroxide was added to the mineral solutions to adjust the final medium to about pH 7·0. Four ml. of solution A and 20 ml. of the silicic acid solution were mixed in Petri dishes (10 cm. diameter). The dishes were left overnight to allow the mixture to solidify. Four ml. silicic acid solution, 0·3 ml. solution B and 0·7 ml. of lignin suspension were mixed in sterile test tubes and poured on the surface of the solidified silica gel in the Petri dishes. When the mixture had solidified the lids of the dishes were removed and replaced by pieces of sterilized filter paper for 2–3 hr. until the surface was dry. Only accidental contaminations occurred on plates prepared in this way.

Measurement of lignin decomposition in soil

Carbon dioxide production. The method described by Petersen (1926) was used but on a smaller scale, with 10 g. portions of soil and 250 ml. bottles. Air-dry loam soil (pH 7·2) was thoroughly mixed with 0·1 and 0·25 % (w/w) of lignin in a mortar; 18 % (v/w) of water containing 0·1 % (w/v) NH_4NO_3 was added afterwards.

The number of lignin-decomposing organisms. Portions of air-dry soil which had passed through a 2 mm. sieve were mixed thoroughly in a mortar with different carbon sources and transferred to 250 ml. bottles; 16–18 % (v/w) of water containing 0·1 % (w/v) NH_4NO_3 was added. The bottles were closed with perforated cork stoppers and incubated at 25°. Ten g. portions of soil were removed from the bottles after about 2 weeks of incubation and shaken in 100 ml. portions of sterile water; 0·5 ml. of a suitable dilution, in most cases 1/5000, was added to the surface of a silica gel + lignin plate and spread by means of a glass rod. The lids of the Petri dishes were replaced by pieces of filter paper until the surface was dry. Transparent spots became visible on the plates after incubation at 25° for about 2 weeks. The spots were counted after incubation for a month.

Isolation of lignin-decomposing bacteria

Lignin-decomposing organisms picked from a transparent spot on a silica gel + lignin plate, inoculated with a soil suspension as described above, were enriched by one or two replatings on the same medium. A spreading was finally performed on agar medium GN. All types of organisms which appeared on this plate after incubation for 2–3 weeks were isolated and tested separately by streaking on silica gel + lignin medium. Several bacteria able to produce a transparency on the lignin medium were isolated in this way. The purity of the isolates was checked by cultivation on nutrient agar, nutrient broth and medium GP alternating with cultivation on silica gel + lignin medium.

Microscopic examinations

The lignin particles. A small square of the surface layer of the silica gel + lignin
medium was removed and placed in a mixture of equal parts 6 N-HCl and 2 % (w/v)
phloroglucinol in ethanol. The square was removed from the solution after a few
minutes, when the lignin particles were intensively stained. After a short drying
on a piece of filter paper the square was placed in a small drop of paraffin oil on
a coverslip. This was mounted on a slide with a circular concavity filled with paraffin
oil so that the square was hanging down immersed in the oil. The coverslip was
sealed to the slide with paraffin and the preparation examined under the microscope.

The microbial growth. Squares of the silica gel + lignin layer were removed and
placed in phenol aniline blue (Jones & Mollison, 1948) for 1 hr., then in water for
15 min. The squares were dried and embedded in paraffin oil as described above.

Quantitative determination of the decomposition of lignin

Silica gel + lignin plates were inoculated with bacteria isolated as described
above. A suspension (0·2 ml.) of the bacteria in water was spread over the surface
by a glass rod. Inoculation with a mixed culture was performed with 0·5 ml. of
suspension of surface material from transparent silica gel + lignin plates. The plates
were incubated at 25° for 6–8 weeks. Uninoculated control plates were incubated
in the same way. At the end of the period of incubation all material was removed
from the dishes, air-dried, ground thoroughly in an agate mortar and extracted for
24 hr. with 96 % (v/v) ethanol in water in a Soxhlet apparatus. The ethanol was
evaporated from the extracts in a current of air and replaced by water which was
left for 24 hr. Material insoluble in water was filtered off in an ignited and weighed
filter crucible (A 1), washed thoroughly with water, dried at 80° and weighed. This
material was undecomposed lignin or residual lignin (RL). Before the chemical
investigations residual lignin was dissolved in dioxan and precipitated by adding
ether.

The silicate material which had been extracted with ethanol was dried in air and
extracted with water at room temperature as long as the extract was brown
coloured, then with 0·1 N-sodium hydroxide in the same way. The extracts were
combined, 2 N-HCl was added to get an acid concentration of about 0·1 N. The
resulting brown precipitate was removed by centrifugation and washed with 0·01
N-HCl. The material was again dissolved in 0·2 N-NaOH, reprecipitated and washed
as described, then dried *in vacuo* and finally at 80°, and weighed. This material was
the oxidized lignin precipitable (OLP).

The supernatant fluids from the precipitations, which were more or less brown
coloured, were combined and neutralized with N-NaOH. Charcoal (British Drug
Houses Ltd. for decolorizing purposes, thoroughly washed with deionized water)
was added, 3 g. to 100 ml., and left for about 2 hr. with periodical stirring. The
charcoal was then filtered off and washed with water. Acetone containing 10 %
(v/v) water was added and a brown coloured material was displaced from the
charcoal. The brown acetone extract was evaporated to dryness in air, the residue
dried at 80° and weighed. This material, which was a brown powder, formed the
oxidized lignin non-precipitable (OLNP).

Analytical methods

Hydrolysis of lignin and its decomposition products with 6 N-HCl. The material (5–15 mg.) was allowed to swell with 0·2–0·4 ml. 6 N-HCl at room temperature in a glass tube. Hard particles were disintegrated with a glass rod 1·0 ml. of 6 N-NCl was then added and the tube was sealed and heated at 105° for 18 hr. Undissolved material was separated from the hydrolysate by centrifugation, washed thoroughly with water, dried at 100°, weighed and total nitrogen determined. The combined hydrolysate and washings were evaporated to dryness *in vacuo*, 0·5 ml. water added and the evaporation repeated. A suitable amount of water was added and α-amino nitrogen determined in a sample; the remainder was used for paper chromatography.

Determination of Klason lignin. This was carried out by treatment with 72 % (w/w) H_2SO_4 as described by Björkman (1957).

Total nitrogen was determined by a micro-Kjeldahl method with $SeO_2 + CuSO_4$. $5H_2O + K_2SO_4$ (1 + 1 + 8) as catalyst.

α-Amino acid nitrogen was determined by the titrimetric ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941).

Methoxyl ($—OCH_3$) was determined by the semimicro method of Vieböck & Schwappach as described by Brauns (1952, p. 744).

Ash. Analytical figures are not corrected for ash content, this was not determined because of the small amounts of material available.

Amino acids. These were detected by paper chromatography on Whatman paper no. 1 with the descending technique. The chromatograms were run in one direction with *n*-butanol + acetic acid + water (4 + 1 + 5, v/v) and in the other direction with phenol + water (80 + 20). The dried chromatograms were sprayed with 0·5 % (w/v) ninhydrin dissolved in water-saturated *n*-butanol containing a little acetic acid. The ninhydrin-positive spots were identified by comparison with spots produced by pure amino acids.

Phenols. These were observed on paper chromatograms developed in one direction with *n*-butanol + acetic acid + water (4 + 1 + 5, v/v). The dried chromatograms were examined in ultraviolet light and fluorescent areas encircled with a pencil, then sprayed with an aqueous solution of 1 % (w/v) $FeCl_3 + 1$ % (w/v) $K_3Fe(CN)_6$ (Coulson, Davies & Lewis, 1960).

Sugars. These were detected on paper chromatograms developed in one direction with *n*-butanol + acetic acid + water (4 + 1 + 5, v/v). The dried chromatograms were sprayed with aniline phthalate or dipped in alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950).

RESULTS

Decomposition of lignin in soil

Carbon dioxide production. Addition of lignin to soil resulted in an increased production of CO_2 (see Fig. 1). Addition of 0·25 % (w/w) lignin caused an increase of 1·6 mg. CO_2/g. dry soil during a period of 74 days. This corresponds to about 29 % of the carbon added as lignin.

Number of lignin-decomposing micro-organisms. Inoculation of silica gel + lignin plates with suspensions of soil resulted in a large number of transparent spots when lignin or lignin-containing plant material had been added to the soil. Plate 1,
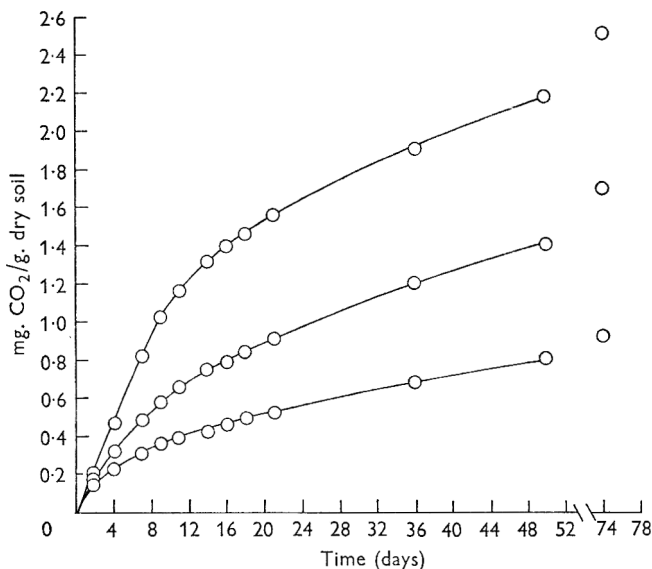
Fig. 1. Carbon dioxide production from soil after addition of 0·25 % (w/w) lignin (upper curve); 0·1 % (w/w) lignin (middle curve); no addition (lower curve).

fig. 2, shows a plate inoculated with 0·5 ml. of a suspension of soil (diluted 1/5000) enriched with 1 % (w/w) powdered wheat straw. Table 1 gives the number of spots observed on plates inoculated with suspensions of soil enriched with a variety of organic materials. The xylan was prepared as described by Sørensen (1957) and the cellulose as described by Norman & Jenkins (1933).

Table 1. *Number of transparent spots which appeared on silica gel + lignin plates after inoculation with suspensions of soil enriched with various carbon sources.*

Inoculation with 0·5 ml. soil suspension diluted 1/5000 except for lignin, where the dilution was 1/25000.

Carbon source	No. transparent spots
Lignin	65
Wheat straw	108
Wood flour	100
Cellulose from wood flour	5
Xylan from wood flour	6
Starch	5
Glucose	14
Xylose	1
Tannin	0
Vanillin	0

A xylan preparation from wheat straw induced a number of transparent spots. This preparation contained, however, 8–10 % lignin-like material not hydrolysed during treatment with 72 % (w/w) sulphuric acid at room temperature followed by boiling under reflux for 5 hr. with 0·5 N-H_2SO_4. It is probably this part of the material which was responsible for the enrichment of the micro-organisms producing the transparent spots.

Decomposition of lignin by pure cultures of bacteria

From transparent spots on silica gel + lignin plates a number of bacteria were isolated which in pure culture produced a similar transparency. Plate 1, fig. 1, shows the transparency produced by a mixed culture (L-15) and a pure culture (*Pseudomonas*-14).

Addition of phloroglucinol + HCl to such plates resulted in a weak coloration of the transparent areas, whereas the other parts of the plate yielded the purple coloration, typical for lignin. This indicated that part of the lignin inside the transparency had disappeared or was unable to yield the colour. Microscopic examination of the surface layer inside and outside the transparent areas showed that only the larger lignin particles were present in the transparent areas, most of the small particles having disappeared. Plate 1, figs. 3 and 4, shows photomicrographs of the surface layer outside and inside a transparent spot.

The surface layer of the silica gel + lignin medium was examined under the microscope after staining with phenol + aniline blue which stained the bacteria but not the lignin particles. Plate 1, fig. 6, shows the fringe of a transparent spot produced by *Pseudomonas*-14. The dark blots are lignin particles surrounded by bacteria. Pl. 1, fig. 7, shows *Pseudomonas*-14 surrounding a lignin particle.

The first indication of a transparency became visible 3–4 days after inoculation and extended then slowly until the medium became too dry. The silica gel + lignin plates seen on Pl. 1, fig. 1, had been incubated for about 1 month after inoculation.

The lignin-decomposing micro-organisms isolated during this work were in all cases bacteria. Fungi and actinomycetes occurred, however, on the lignin medium after inoculation with soil suspensions. Several such organisms were isolated and tested separately on silica gel + lignin medium but none of these isolates produced any transparency; the growth of the isolates on this medium was very feeble.

Pl. 1, fig. 5, shows micro-organisms from silica gel + lignin medium inoculated with the mixed culture L-15. This culture had been replated on lignin medium for about a year. The organisms seen on this photo are considered to be typical for the whole plate.

Description of some lignin-decomposing bacteria

The lignin-decomposing bacteria isolated in pure culture during this work were of two types: (1) non-endospore forming straight rods, $1 \cdot 3 – 1 \cdot 5 \times 0 \cdot 5 \mu$, motile with one or two polar flagella, Gram-negative, aerobic; (2) non-endospore forming straight rods, $1 \cdot 0 – 1 \cdot 2 \times 0 \cdot 6 \mu$, non-motile, Gram-negative, aerobic.

Some cultural, nutritional and biochemical characters. The tests were performed in 5 ml. portions of media containing the respective carbon sources in 0·5 % (w/v) concentration and nitrogen sources in 0·1 % (w/v) concentration besides the basal mineral solution. Soil extract (25 %, v/v) was added in some cases. Incubation was at 25° and observation was over a period of a month.

Growth on nutrient and GP agar. Colonies greyish white (group 1) or yellow (group 2), circular, convex, edge regular, surface smooth and shining, diameter after 1 month 1·0–1·5 mm. Growth nonspreading. No water soluble pigment.

Nutrient broth and GP broth. Turbidity visible 1–2 days after inoculation, growth

homogeneous, no soluble pigment, no odour. A deposit of cells 3–4 weeks after inoculation.

Micro-aerophilic conditions. Nutrient broth and GP broth boiled before inoculation and covered by a layer of paraffin oil, a faint turbidity was visible 3–4 days after inoculation; it did not increase during prolonged incubation.

Optimal temperature for growth. Best growth at 30°; no growth at 37°.

Effect of initial pH *of the medium.* No difference in growth rate was observed within the range pH 5·3–7·8.

Growth in defined medium. Basal mineral solution+ammonium nitrate and sodium citrate or sodium acetate: no growth. Addition of soil extract had no effect.

Utilization of carbohydrates. Slight acid formation but no gas from glucose, mannose, xylose, arabinose and salicin. Best growth on glucose and mannose; no or very slight growth on galactose, levulose, sucrose, maltose, lactose, mannitol, glycerol. The sugar solutions were sterilized by filtration. The nitrogen source was peptone.

Utilization of polysaccharides. Strips of filter paper partly immersed in basal mineral solution+soil extract, peptone or ammonium nitrate: no utilization. Starch (soluble), mannan and xylan (Sørensen, 1957) added to GP-agar instead of the glucose: no or very slight utilization.

Potato. Faint growth of a brownish colour (group 1), yellow growth (group 2).

Utilization of nitrogen sources. Peptone and glutamic acid yielded the best growth, ammonium and asparagine medium growth, slow growth with nitrate.

Catalase. Positive.

Gelatine. No or very slight liquefaction.

Milk. No changes of the milk visible.

Formation of nitrite from nitrate. Negative.

Formation of ammonia from peptone. Positive, moderate.

According to *Bergey's Manual* (1957) group 1 belongs to the genus *Pseudomonas* and group 2 to the genus *Flavobacterium*. A further classification according to the descriptions given by *Bergey's Manual* was not possible. The bacteria were isolated from loam soil (pH 6·5–7·5) representing agricultural, garden and forest soils. Six isolates of the *Pseudomonas* sp., three isolates of the *Flavobacterium* sp. The bacteria belonging to the last genus gradually lost in pure culture the ability to decompose lignin.

Quantitative determination of the decomposition of lignin

Tables 2 and 3 summarize the results of the quantitative determinations of the lignin decomposition and the analyses carried out on the extracted material. The nitrogen sources added to the silica gel+lignin medium were peptone (Table 2) or nitrate (Table 3). The figures in the upper part of the two tables indicate the % of the lignin originally present which was recovered as residual lignin (RL) or as oxidized lignin (OLP, OLNP). A sample of lignin from a water suspension, 2 months old, was mixed with quartz sand and air-dried and finally subjected to the extraction procedure used for the silica gel+lignin samples. It is seen (Table 3) that almost 97 % of the lignin was recovered by this procedure.

The silica gel+lignin plates were incubated for 6–8 weeks at 25°. The biological decomposition took place during the first 3–4 weeks as judged by the increasing transparency of the medium. Uninoculated sterile control plates were analysed

shortly after preparation or incubated in the same way as the inoculated plates. The later stages of the incubation when the medium dried out more or less were characterized by the development of a brown colour both in inoculated and un-inoculated plates. This coloration is considered to be the result of an autoxidation of a part of the lignin. The products were soluble in water but insoluble in ethanol, precipitable (OLP) or non-precipitable (OLNP) on addition of acid. A part of the non-precipitable fraction was able to cross a dialysis membrane.

Table 2. *Material recovered from inoculated and uninoculated silica gel + lignin plates and the results of the chemical analyses performed on this material*

0·05 % (w/v) peptone was added as a nitrogen source.

	Mixed population L-15		*Pseudomonas*-14		Uninoculated*		Uninoculated†	
Recovered as lignin (RL), %	21·6		26·8		52·9		61·4	
Recovered as oxidized lignin (OLP), %	26·9		30·7		29·1		22·5	
Recovered total, %	48·5		57·5		82·0		83·9	
	RL	OLP	RL	OLP	RL	OLP	RL	OLP
Nitrogen, %	0·25	2·05	0·26	2·53	0·32	2·54	0·31	2·65
Insoluble residue from hydrolysis with 6N-HCl, %	84·4	70·0	90·4	70·5	87·3	80·5	85·7	76·9
Nitrogen in residue as % of original matter	0·16	0·57	0·16	0·56	0·19	0·51	0·20	0·59
Nitrogen in residue, %	0·19	0·81	0·17	0·80	0·22	0·63	0·23	0·76
Nitrogen hydrolysed, %	36·5	72·2	39·5	77·7	39·0	80·1	36·5	77·8
α-Amino acid nitrogen as % of original matter	0·08	1·01	0·08	1·03	0·05	1·19	0·05	1·39
α-Amino acid nitrogen as % of total nitrogen	32·0	49·5	30·8	40·6	15·6	47·0	16·1	52·5
α-Amino acid nitrogen as % of hydrolysed nitrogen	89·0	68·0	80·0	52·3	38·5	58·5	45·5	67·5

* Incubated in the same way as the inoculated plates.
† The medium was dried immediately after preparation.

It is seen that about the same % of oxidized lignin was recovered from the sterile plates as from the inoculated ones indicating that the transformation of the lignin to the dark-coloured compounds was accomplished by non-biological means. Similar amounts were recovered from plates dried immediately after preparation as from sterile plates allowed to dry slowly during an incubation period of 6–8 weeks (Table 2). This indicated that the transformation occurred during the drying process. No oxidation occurred in the suspension of lignin in water. Ammonia appeared in some way to protect the lignin against the autoxidation. Larger amounts of lignin and less oxidized lignin were recovered when 0·1 % (w/v) NH_4Cl was added to the medium. This was not investigated further, but nitrate or peptone were added as nitrogen source in experiments where a quantitative determination of lignin and oxidized lignin was performed.

The biological decomposition of lignin. A comparison between the figures indicating the % of material recovered totally and as residual lignin from inoculated and

Table 3. *Material recovered from inoculated and uninoculated silica gel + lignin plates and the results of the chemical analyses performed on this material*

0.1% (w/v) KNO_3, added as nitrogen source.

	Mixed population			Pseudomonas-14			Uninoculated			Original lignin	
	RL	OLP	OLNP	RL	OLP	OLNP	RL	OLP	OLNP	A*	B*
Recovered as lignin (RL), %	25.0			33.4			59.1			96.1	—
Recovered as oxidized lignin (OLP), %	17.0			19.6			13.2			0.6	—
Recovered as oxidized lignin (OLNP), %	7.8			9.6			7.5			0.0	—
Recovered total, %	49.8			62.6			79.8			96.7	—
Nitrogen, %	0.25	0.62	0.55	0.21	0.58	0.64	0.11	0.39	0.69	0.18	0.1
Insoluble residue from hydrolysis with 6 N-HCl, %	88.9	84.9	40.0	87.0	86.5	17.4	86.7	89.4	14.8	85.3	89.3
Nitrogen in residue as % of original matter	0.14	0.31	0.15	0.12	0.31	0.12	0.07	0.26	0.09	0.12	0.1
Nitrogen in residue, %	0.16	0.37	0.37	0.14	0.35	0.67	0.08	0.27	0.61	0.15	0.1
Nitrogen hydrolysed, %	43.7	49.6	72.8	44.3	47.7	81.6	38.7	31.7	87.0	36.3	33.5
α-Amino acid nitrogen as % of original matter	0.05	0.18	0.19	0.04	0.16	0.12	0.01	0.07	0.12	0.03	0.0
α-Amino acid nitrogen as % of total nitrogen	20.0	29.0	34.6	19.1	27.6	18.8	9.1	18.0	17.4	16.7	17.6
α-Amino acid nitrogen as % of hydrolysed nitrogen	45.5	58.0	47.5	44.5	59.2	23.1	25.0	54.0	20.0	50.0	50.0
—OCH_3, %	11.1	10.0	—	12.5	9.8	—	13.9	11.3	—	12.6	12.8

* A. An amount of original lignin from a suspension in water dried on quartz sand and extracted with ethanol etc.; B, Analytical figures for original lignin.

sterile plates respectively, showed a difference which is ascribed to a biological decomposition. Table 3 shows that 49·8 % was recovered totally from plates inoculated with a mixed population, 62·6 % from plates inoculated with *Pseudomonas*-14 and 79·8 % from the sterile control plates. The figures indicating recovered lignin are 25·0 %, 33·4 % and 59·1 %, respectively. This indicates that about 30 % of the lignin was decomposed by the mixed population and about 20 % by the *Pseudomonas* sp. The figures given in Table 2 for medium containing peptone as nitrogen source indicate a degree of decomposition of the same order.

Chemical analyses of lignin and oxidized lignin

The residual lignin and oxidized lignin extracted from the silica gel + lignin medium during the determination of the decomposition of lignin were subjected to various analyses. The results are summarized in Tables 2 and 3.

Nitrogen. Residual lignin was only slightly influenced by the nitrogen source present in the medium from which it was extracted. The content of total nitrogen varied from 0·11 % in a sample from uninoculated nitrate-containing medium to 0·32 % in a sample from uninoculated peptone-containing medium.

The nitrogen content of oxidized lignin was, however, strongly influenced by the nature of the nitrogen compounds present. The total content varied from 0·39 % in a sample from uninoculated nitrate-containing medium to 2·65 % in a sample from uninoculated peptone-containing medium. The total nitrogen content of the insoluble residue from the same samples varied from 0·27 to 0·76 %. The α-amino acid nitrogen content varied from 0·07 to 1·39 %. The nitrogen content of the non-precipitable fraction of oxidized lignin was of the same order as that of the precipitable fraction, but a larger percentage was susceptible to hydrolysis. These figures indicate that oxydized lignin readily complexes with nitrogenous compounds present in peptone especially amino acids. Difco Bacto peptone contains 16·2 % total-N, 15·4 % peptone-N and 3·2 % free amino-N (Sykes, 1956).

Amino acids. Hydrolysis with 6 N-HCl released a number of amino acids from lignin and oxidized lignin. Fifteen to sixteen ninhydrin-positive spots appeared on the paper chromatograms. The most intense of these spots were identified as alanine, glycine, leucine, valine, serine, threonine, glutamic acid, aspartic acid, lysine, arginine, proline. The qualitative amino acid composition appeared to be identical in the hydrolysates of the various fractions but the quantitative composition appeared to differ; in particular the oxidized lignin extracted from peptone-containing medium was different from the other preparations.

Phenols. The chromatograms of the hydrolysates showed under ultraviolet radiation several fluorescent spots with R_F-values ranging from 0·07 to 0·9. Spraying with the ferric chloride + ferricyanide reagent resulted in green or blue spots mainly identical with the fluorescent spots. Hydrolysates of original and residual lignin and of precipitable and non-precipitable oxidized lignin showed almost the same fluorescent spots on the paper chromatograms. Identification of the spots with known compounds was not attempted.

Carbohydrates. The original lignin contained some carbohydrate material; glucose, arabinose, xylose and uronic acids were released during treatment with 72 % sulphuric acid as described by Björkman (1957).

The bacterial decomposition of lignin was not a selective utilization of the

carbohydrate impurities. This was shown by hydrolysis of residual lignin from a medium where a bacterial decomposition had taken place. The constituents mentioned above were present in such hydrolysates and, judging by the intensity of the spots, were in the same relative concentrations.

Methoxyl. The —OCH_3 content of residual lignin and oxidized lignin extracted from the nitrate-containing medium is given in Table 3. It is seen that residual lignin from the sterile medium had a —OCH_3 content larger than that of the original lignin. This might be the result of a removal of impurities. The methoxyl content of the oxidized lignin was 10–20 % lower than that of residual lignin.

DISCUSSION

The results of this work indicated that isolated lignin can be decomposed by soil bacteria. Addition of lignin-containing plant material to soil samples resulted in the enrichment of a special population of micro-organisms able to attack the isolated lignin. This indicated that the preparation represented a natural part of plant material and not an artefact produced during the extraction and purification. The preparation contained, however, some protein and carbohydrate material but the bacterial decomposition was not a selective utilization of these constituents, as shown by the chemical analyses of the residual material.

The non-biological transformation of lignin into brown coloured water soluble substances might be catalysed by the presence of silicate. Autoxidations appear to be strongly influenced by the environment; Pratt & Trapasso (1960) observed an autoxidation of a variety of organic solids (e.g. anthracene) by passing air through a mixture of the powdered compound and alumina. Ziechmann (1959) reported that the oxidation of polyhydroxybenzenes proceeded slowly at a neutral reaction in the presence of silicic acid. The chemical analyses of the brown coloured substances indicated a similarity with the lignin and they might represent slightly altered constituents of the lignin molecule. Their ability to react with organic nitrogen compounds was, however, much larger than that of the lignin proper. The complexes which resulted from this reaction had a certain resemblance to the soil humic acid investigated by Bremner (1955) in so far as the nitrogen content, the % of amino acid nitrogen and the amino acids detected in the hydrolysates were similar. This result seems to support the view that soil humic acid complexes result from a reaction between more or less altered lignin originating from the plant material and nitrogen compounds originating from the protein synthesized by the soil micro-organisms. This point of view has been put forward by Gillam (1940) who found evidence that the non-nitrogenous fraction of humic acid consisted of a modified lignin. Mattson & Koutler-Andersson (1943) observed a relation between autoxidation of lignin and fixation of ammonia and they assumed that the humus complexes of soil were derived from lignin by such processes. Handley (1954) found that protein became resistant to microbial attack after reaction with substances, probably tannins, leaching from the litter on top of the soil; such a process was considered to be an important factor in the formation of mor.

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EXPLANATION OF PLATE 1

Fig. 1. Transparency on silica gel + lignin plates after inoculation with lignin-decomposing micro-organisms. The left-hand plate inoculated with a mixed culture (L-15); the right-hand plate inoculated with a pure culture (*Pseudomonas-14*). Incubation for a month.

Fig. 2. Transparent spots on a silica gel + lignin plate inoculated with 0·5 ml. of a suspension (diluted 1/5000) of soil enriched with 1 % (w/w) powdered wheat straw. Incubation for a month.

Fig. 3. Photomicrograph showing the lignin particles in the surface layer of an uninoculated silica gel + lignin plate stained with phloroglucinol + HCl. × 400.

Fig. 4. Photomicrograph showing the lignin particles in the surface layer of a silica gel + lignin plate rendered transparent by the growth of *Pseudomonas-14*. Staining with phloroglucinol + HCl. × 400.

Fig. 5. Photomicrograph of the surface of a silica gel + lignin plate inoculated with a mixed culture (L-15). Staining with phenol + aniline blue. × 1000.

Fig. 6. Photomicrograph of the fringe of a transparent spot on a silica gel + lignin plate inoculated with *Pseudomonas-14*. Staining with phenol + aniline blue which stain the bacteria but not the lignin particles. The blots are lignin particles surrounded by bacteria. × 640.

Fig. 7. Photomicrograph showing *Pseudomonas-14* surrounding a lignin particle. Staining with phenol + aniline blue. × 1000.
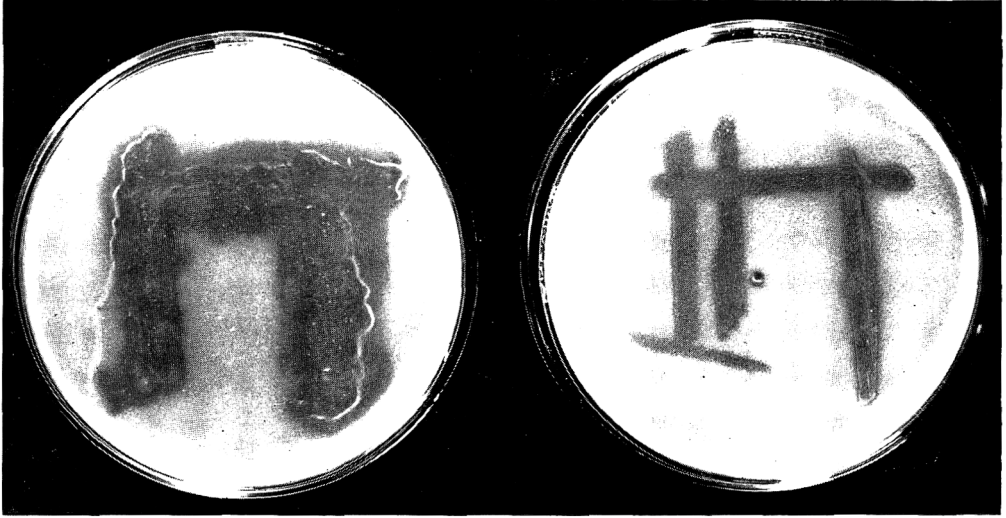
Fig. 1
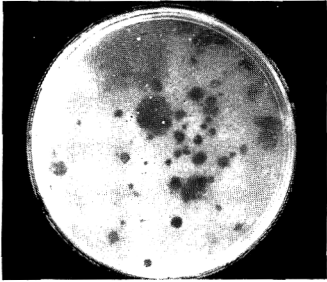
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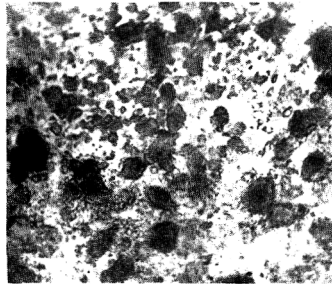
Fig. 3
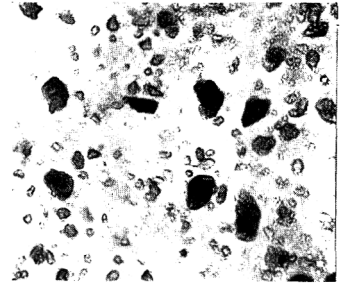
Fig. 4

Fig. 5

Fig. 6

Fig. 7

H. SØRENSEN
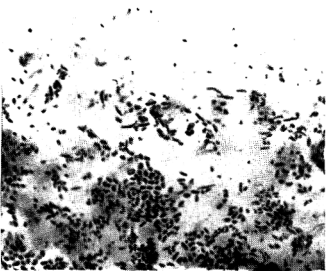
Quantitative Aspects of the Intestinal Yeast Flora of Swine

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SUMMARY

Minimum numbers of viable organisms of the yeast species occurring in the digestive tract of 57 pigs were determined in samples from six sites in each animal (stomach, three sites in the small intestine, caecum, rectum). In 27 animals (47·4 %) *Candida slooffii* was found in one or more sites in numbers ranging from less than 300 to 9,000,000 viable organisms/g. wet sample. The trend was an increase in numbers towards the anus, with highest counts in the rectum and next highest counts in the caecum. The food eaten influenced the numbers of *C. slooffii*; food rich in starch apparently increased, while food rich in cellulose and proteins decreased, these numbers. The pig appears to constitute a suitable host for the obligate intestinal saprophyte *C. slooffii*. Other yeast species found at one or more sites in numbers above 100/g. wet sample (in brackets the number of positive animals) were: *Candida krusei* (8); *Saccharomyces cerevisiae* (6); *Pichia membranaefaciens* (6); *C. albicans* (3); *S. tellustris* (*C. bovina*) (2); *S. chevalieri* (2); *S. oleaceus* (2); *S. carlsbergensis* (2); *C. parapsilosis* (2); *C. tropicalis* (2); *S. italicus* var. *melibiosi* (1); *S. steineri* (1); *C. utilis* (1); *Torulopsis glabrata* (1); not identified (1). Analysis of the counts of the three most frequent species showed an increase in the anal direction. It is concluded that *C. krusei*, *P. membranaefaciens* and *S. cerevisiae* are facultative intestinal saprophytes of swine.

INTRODUCTION

During the past few years several related yeast species, well adapted to life in the digestive tract of warm-blooded animals, have been described (*Torulopsis pintolopesii*, Van Uden, 1952; *Candida bovina*, Van Uden & Carmo-Sousa, 1957*a*; *Saccharomyces tellustris*, Van der Walt, 1957; *C. slooffii*, Van Uden & Carmo-Sousa, 1957*b*). Nutritional fastidiousness and a narrow temperature range with a high minimum are characteristic for this group. *C. slooffii* grows only between 28° and 44° and requires biotin, inositol, nicotinic acid, thiamine and, under certain circumstances (Kreger-van Rij, 1958), pantothenate. Originally isolated from 6 out of 252 horses (Van Uden & Carmo-Sousa, 1957*b*), *C. slooffii* was subsequently found also in the digestive tract of 121 out of 250 pigs (Van Uden, Carmo-Sousa & Farinha, 1958). This high incidence (48·4 %) suggested that the pig rather than the horse might be a natural host for *C. slooffii*. In the present study we have tried to obtain quantitative information about *C. slooffii* and other yeast species which, as previous qualitative work showed (Van Uden *et al.* 1958), occur in the digestive tract of swine.

METHODS

Fifty-seven swine were sampled in the abattoir of Lisbon. Samples were taken from the contents of the digestive tract at the following sites: stomach (S); small intestine at a point one-third of its total length from the pylorus (I ⅓); small intestine at a point two-thirds of its total length from the pylorus (I⅔); small intestine a few cm. oral of the caecum (I⅔); caecum (C); rectum (R).

Table 1. *Quantitative occurrence of* Candida slooffii *in the digestive tract of swine*

Serial number of pig	Site of sample					
	S*	I⅓	I⅔	I⅔	C	R
	Counts of *C. slooffii* (organisms/g. wet intestinal contents)					
1	< 300	< 300	< 300	330	< 300	< 300
3	380	480	< 300	< 3,000	< 3,000	< 300
4	840	< 300	< 300	13,500	65,000	270,000
6	360	420	370	< 300	< 300	< 300
7	0	0	0	0	< 300	0
11	1,030	< 300	< 300	4,450	< 300	440
12	< 300	0	0	0	< 300	< 3,000
13	< 300	< 300	< 300	< 300	< 300	< 300
14	1,230	0	< 300	< 300	224,000	9,000,000
15	< 300	0	0	< 300	830	1,330
19	0	0	0	0	1,260	93,000
20	0	0	0	0	0	330
21	0	0	0	0	6,750	6,000
22	< 300	< 300	< 300	420	139,000	15,500
23	< 300	< 300	< 300	< 300	1,100	2,490
24	< 300	< 300	< 300	980	3,500	420
25	44,250	2,130	2,060	12,200	329,500	525,000
26	8,450	3,160	3,420	38,500	121,000	80,500
31	< 300	< 300	2,830	520	212,000	660,000
32	< 300	< 300	0	< 300	< 300	470
33	< 300	< 300	< 300	540	< 300	0
34	0	0	0	0	< 300	< 300
35	0	0	0	< 300	< 300	0
43	< 300	0	0	< 300	5,500	21,400
44	6,770	14,160	10,650	4,470	2,640	720
45	1,060	< 300	2,080	19,900	965,000	1,925,000
57	920	1,910	2,220	6.600	7,800	5,750

< 300 = less than 30 colonies on the 1/10 dilution plates.
< 3000 = less than 30 colonies on the 1/100 dilution plates, the 1/10 plates having been lost.
* S = Stomach; I⅓ = small intestine at a point one third of its total length from the pylorus; I⅔ = small intestine at a point two thirds of its total length from the pylorus; I⅔ = small intestine a few cm. oral of the caecum; C = caecum; R = rectum.

Portions (1 g.) of the samples were suspended in 10 ml. sterile distilled water, shaken during 10 min. and serial dilutions plated in 1 ml. amounts in a medium of the following composition: glucose, 2 % (w/v); yeast extract (Difco), 0·5 % (w/v); peptone, 1 % (w/v); agar, 2 % (w/v); penicillin, 60 units/ml.; streptomycin, 100 units/ml. After incubation at 37° for 48–72 hr., the numbers of yeast colonies were recorded according to macroscopic and microscopic morphology. Representants of each type were subcultured for identification. The yeast isolates were identified by the methods of Lodder & Kreger-van Rij (1952), Wickerham (1951) and Van Uden & Farinha (1958).

RESULTS

We found *Candida slooffii* in the digestive tract of 27 of the 57 pigs studied, an incidence of 47·4 %, which is near the value 48·4 % found during a previous qualitative survey (van Uden *et al.* 1958). Thus *C. slooffii* appears to occur in about 50 % of the digestive tracts of the pigs examined. This is an unusually high incidence for a single yeast species in the digestive tract of healthy unselected individuals of a warm-blooded host species and is inferior only to the incidences found for *Saccharo-*

Table 2. *Yeasts other than* Candida slooffii, *occurring in the digestive tract of swine in numbers above* 100/g. *wet intestinal contents*

Serial number of pig	Site of sample					
	S*	I⅓	I⅔	I¾	C	R
	Counts of yeast (organisms/g. wet intestinal contents)					
2	0	0	0	0	0	A < 300
3	0	0	0	H 9,400	H 7,400	H < 300
8	0	0	0	0	0	A 3,000
						C 4,000
9	0	0	0	0	0	A 5,100
						C 2,040
13	0	0	0	0	0	A < 300
16	0	0	0	0	0	F < 300
17	A† < 300	0	0	0	A < 300	A 12,100
	L < 300					F 15,800
19	C 340	C < 300	C < 300	C < 300	0	0
20	A < 300	0	0	A 2,340	A 2,790	0
				M 700	N 350	
27	A 2,310	A < 300	A < 300	A < 300	A < 300	C 2,160
		C < 300	D < 300	G < 300		B < 300
		J < 300		D 330		E < 300
34	D 330	D < 300	D < 300	D < 300	0	C < 300
						B < 300
35	A < 300	0	0	B < 300	B < 300	J 12,000
39	0	0	0	0	0	E 52,500
40	B 20,300	B 450	B 3,300	B 4,500	B 300,000	B 1,855,000
41	0	0	0	0	0	B < 300
						C < 300
						I < 300
						P < 300
42	B 30,000	0	B 30,000	B 30,000	B 435,000	B 2,148,000
						R 201,000
44	D 8,270	D 7,720	0	D 7,270	D 1,760	D < 300
		G 3,860				Q < 300
47	0	0	0	0	0	P 16,500

* S = Stomach; I⅓ = small intestine at a point one-third of its total length from the pylorus; I⅔; small intestine at a point two-thirds of its total length from the pylorus; I¾ = small intestine a few cm. oral of the caecum; C = caecum; R = rectum.

† Key to organisms:

A = *Candida krusei*	G = *C. tropicalis*	N = *Torulopsis glabrata*
B = *Pichia membranaefaciens*	H = *S. tellustris*	P = *S. oleaceus*
C = *Saccharomyces cerevisiae*	I = *S. italicus* var. *melibiosi*	Q = *C. bovina*
D = *C. albicans*	J = *S. carlsbergensis*	R = Not identified
E = *S. chevalieri*	L = *S. steineri*	
F = *C. parapsilosis*	M = *C. utilis*	

< 300 = less than 30 but more than 10 colonies on the 1/10 dilution plates.

mycopsis guttulata in rabbits (Shifrine & Phaff, 1958) and *Torulopsis pintolopesii* in mice and rats (Mackinnon, 1959). This indicates a high suitability of swine as hosts for *C. slooffii.*

The minimum numbers of viable organisms of *Candida slooffii*/g. wet intestinal contents differed from animal to animal and from site to site in the same animal, between extremes of 9,000,000 and less than 300 (Table 1). The counts of *C. slooffii* in the digestive tract showed a trend to increase towards the anus; the second highest counts were most frequent in caecal contents, the highest counts in rectal contents (Fig. 1). This shows that *C. slooffii* multiplies while moving through the digestive tract. The *C. slooffii* population reaches its highest density when leaving the digestive tract. This implies some drainage at the pig's expense of the growth factors on which *C. slooffii* is dependent (see introduction).

Table 3. *Incidences in swine digestive tract of yeast species other than* Candida slooffii, *showing more than* 100 *organisms/g. wet intestinal contents, compared with the incidences of yeast species isolated during a qualitative survey*

The figures give percentage of animals which were positive.

Quantitative survey	(%)	Qualitative survey*	(%)
Candida krusei	14·0	*C. krusei*	14·8
Saccharomyces cerevisiae	10·5	*S. tellustris (C. bovina)*	14·0
Pichia membranaefaciens	10·5	*C. albicans*	9·2
C. albicans	5·3	*S. cerevisiae*	8·8
S. tellustris (C. bovina)	3·5	*P. membranaefaciens (C. mycoderma)*	7·6
S. chevalieri	3·5	*C. tropicalis*	6·0
S. oleaceus	3·5	*T. glabrata*	3·2
S. carlsbergensis	3·5	*S. italicus*	0·8
C. parapsilosis	3·5	*C. parapsilosis*	0·8
C. tropicalis	3·5	*S. carlsbergensis*	0·4
S. italicus var. *melibiosi*	1·8	*P. farinosa*	0·4
S. steineri	1·8	*Hansenula angusta*	0·4
C. utilis	1·8	*T. famata*	0·4
Torulopsis glabrata	1·8	*Trichosporon cutaneum*	0·4
Not identified	1·8		

* After Van Uden, Carmo Sousa & Farinha (1958).

Though swine are suitable hosts for *Candida slooffii*, not every animal harbours this yeast at a given time and the numbers or organisms in the positive animals may vary greatly. One factor which interferes with individual host suitability seems to be the diet fed. From our not wholly complete records about the diets of the pigs sampled, the following can be said: (1) most pigs which did not reveal the presence of *C. slooffii* had been kept on a green food diet; (2) most pigs with low numbers of *C. slooffii* throughout the digestive tract had been on a mixed diet, including kitchen refuse; (3) most pigs with high counts for *C. slooffii* had been kept on a grain diet, chiefly maize. These observations suggest that food rich in cellulose (1) and proteins (2) had an adverse effect on intestinal *C. slooffii*, and food rich in starch (3) a stimulatory effect.

In 18 of the 57 pigs, yeast species other than *Candida slooffii* were found in numbers exceeding 100 viable organisms/g. intestinal contents (Table 2). Most of these species had also been found in an earlier qualitative survey (Table 3). *C. krusei,*
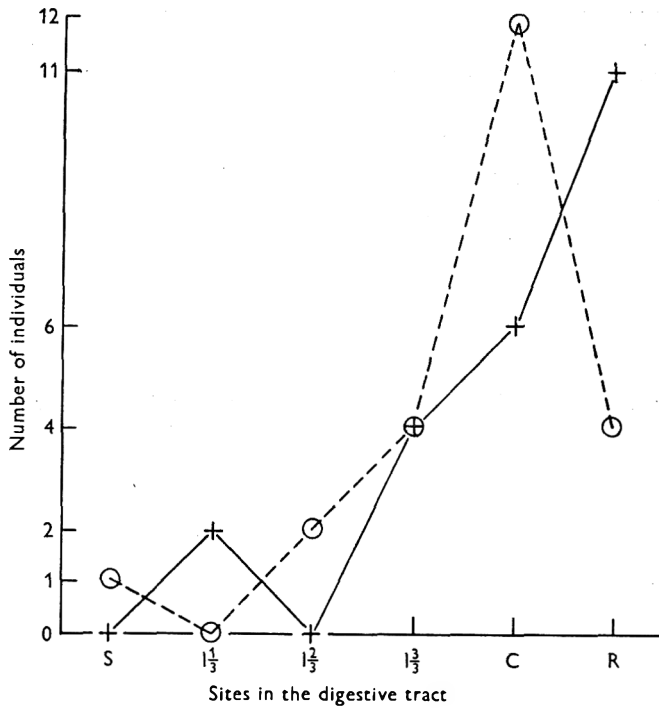
Fig. 1. Frequency of highest and second highest counts for *Candida slooffii* in six sites of the digestive tract of 57 swine. S = stomach; I $\frac{1}{3}$ = small intestine at a point one-third of its total length from the pylorus; I $\frac{2}{3}$ = small intestine at a point two-thirds of its total length from the pylorus; I $\frac{3}{3}$ = small intestine a few cm. oral of the caecum; C = caecum; R = rectum. + = highest counts; ○ = second highest counts.
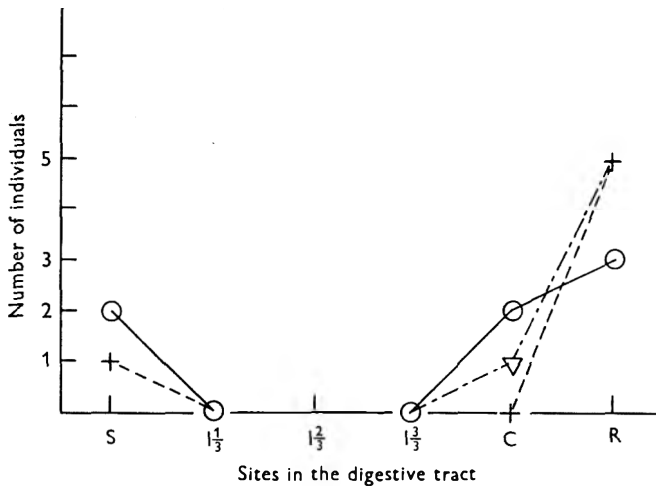
Fig. 2. Frequency of highest counts for *Candida krusei, Saccharomyces cerevisiae* and *Pichia membranaefaciens* in six sites of the digestive tract of 57 swine: S = stomach; I $\frac{1}{3}$ = small intestine at a point one-third of its total length from the pylorus; I $\frac{2}{3}$ = small intestine at a point two thirds of its total length from the pylorus; I $\frac{3}{3}$ = small intestine a few cm. oral of the caecum; C = caecum; R = rectum. ○ = *Candida krusei*; + = *Saccharomyces cerevisiae*; ▽ = *Pichia membranaefaciens*.

Pichia membranaefaciens and *Saccharomyces cerevisiae* were the most frequent species with numbers ranging from more than 100 to more than 2,000,000 organisms/g. wet intestinal contents. These three species are widespread in nature. When they occur in the digestive tract they are therefore either passers-by or facultative saprophytes. The latter possibility is the case in the pig's digestive tract as is shown in Fig. 2; the highest counts for those three species were more frequent in the rectum than in other parts of the digestive tract, which shows their capacity of multiplication in this environment. *S. tellustris* (*C. bovina*), *C. albicans* and *Torulopsis glabrata*, well known as obligate saprophytes of warm-blooded animals, do not seem to be particularly well adapted to healthy pigs, as is suggested by their low incidences and low cell numbers (Table 2).

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Studies on a Mutant Strain of *Escherichia coli* which Requires both Methionine and Lysine for Growth

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SUMMARY

A mutant of *Escherichia coli* was isolated which showed a requirement for methionine + lysine when a fairly large inoculum of washed organisms was used. Cystathionine or homocysteine replaced methionine, and diaminopimelic acid replaced lysine; no other amino acid or growth factor was active. The amounts of the amino acids required to promote growth were only about one-tenth of the quantity of methionine and lysine found in the grown organisms. A quantitative study with ^{14}C-glucose and ^{35}S-sulphate confirmed that these amino acids were synthesized *de novo*. Although homoserine would not replace methionine for growth, the presence of unlabelled homoserine suppressed the incorporation of isotope from ^{14}C-glucose into methionine and threonine, indicating that the accepted pathways are operative and that the block in methionine synthesis was in the formation of cystathionine. Small amounts of yeast extract replaced the amino acid requirement and decreased one-thousandfold the size of the inoculum required.

INTRODUCTION

Several reports suggest a relationship between the biosyntheses of methionine and lysine by bacteria. Davis (1952*a*, 1953) described mutants of *Escherichia coli* which, in the presence of traces of aspartic acid, showed an absolute requirement for *p*-hydroxybenzoic acid, which was satisfied by lysine + methionine, and it was suggested that *p*-hydroxybenzoic acid might therefore function in the synthesis of both these amino acids. Work (1955) reported the existence of several double mutants of *E. coli* which required lysine + methionine. Davis (1952*b*) described *E. coli* mutants, with a requirement for diaminopimelic acid or lysine or both, which accumulated threonine (derived from homoserine, a methionine precursor); another mutant utilized threonine as an alternative to diaminopimelic acid. The isolation by the present authors of a mutant of *E. coli* which requires methionine + lysine for growth prompted an investigation of the nature of the metabolic block, since existing information about the biosynthesis of these two compounds indicates no link or common precursor after aspartyl-β-semialdehyde (Fig. 1). This paper reports the results of a quantitative study of the nutrition of this mutant, by using ^{14}C- and ^{35}S-labelled compounds.

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METHODS

Organism. *Escherichia coli* substrain 15 is an auxotroph, derived by ultraviolet irradiation of wild-type *E. coli* 518 by the procedure of Adelberg & Meyers (1953); the mutant exhibits a requirement for methionine + lysine. The mutant was maintained by monthly subculture on papain digest agar (Skerman, 1959) incubated at 37° for 18 hr.

Inoculum. Organisms from an 18 hr. papain digest agar slope culture were suspended in sterile water at a concentration to give an absorption reading of 0·30
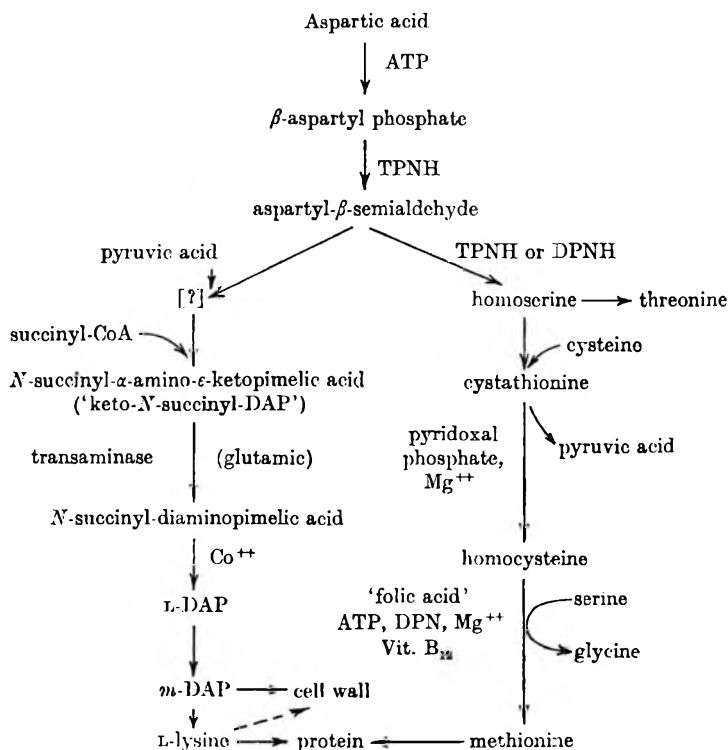
Fig. 1. The biosynthesis of methionine and lysine by bacteria (modified from Gilvarg, 1960).

at 420 mμ on the Unicam spectrophotometer Model SP 600; the organisms were washed twice with sterile de-ionized water and resuspended to a concentration corresponding to an absorption reading of 0·20 (about 7×10^7 organisms/ml.). The short wavelength was chosen to permit accurate measurement of the relatively dilute suspensions used.

Growth tests. Growth tests were made in 'minimal medium C' containing 0·2 % (w/v) glucose (Roberts *et al.* 1955). The test medium was dispensed in 6 in. $\times \frac{3}{4}$ in. test tubes to give 4·0 ml. final volume and autoclaved at 115° for 7 min. Amino acid supplements (sterile solutions) and the inoculum (10 %, v/v) were added after autoclaving. Tests, in duplicate, were incubated for 24 hr. at 37° and the growth response recorded either qualitatively or by optical density measurement. The

organisms in each tube were tested for viability and reversion by streaking a loopful on to minimal medium agar and papain digest agar and incubating for 24 hr. at 37°. Reversions were always to the prototroph, indicating that the double require-ment was due to a single genetic block. For tests on solid medium, plates were prepared from a minimal medium containing washed agar (2 %, w/v) and inoculated with a 5 % (v/v) inoculum of the standard suspension described above. Growth supplements were supplied either by placing a small crystal of solid on the agar or by dipping a sterile 'fish-spine' bead (Bray's Interlocking Insulating Beads, Geo. Bray Co. Ltd., Leicester Place, Leeds, England; size no. 2) in 0·01 M solution and applying to the agar surface. Plates were incubated for 24 hr. at 37°.

Preparation of ¹⁴C- *and* ³⁵S-*labelled cell hydrolysates.* Cultures (50 ml.) grown in minimal medium containing ¹⁴C-glucose 0·5 μc./ml. or ³⁵S-sulphate 0·3 μc./ml. (supplemented with minimal lysine and methionine in the case of the mutant) were harvested after incubation for 24 hr. at 37° and washed in de-ionized water at 3000 g for 10 min. until the washings were free from radioactivity. The organisms were suspended in 5 ml. 6 N-HCl and hydrolysed by heating at 110° for 16 hr. under nitrogen. The hydrolysates were filtered, washed, dried over solid NaOH *in vacuo*, redissolved, dried again and taken up in a measured volume of water. Amino-N was determined by the method of Moore & Stein (1954).

Chromatography. Cell hydrolysates labelled with ³⁵S were subjected to two-dimensional paper chromatography without further treatment. Cell hydrolysates labelled with ¹⁴C were fractionated in ion exchange columns into the basic amino acid fraction and the neutral + acidic amino acid fraction (Thompson, Morris & Gering, 1959). The fractions were concentrated *in vacuo* and the amino-N content of each determined. Ammonia, which interferes with this determination, was removed by the addition of borate buffer in methanol to each sample for assay, which was then dried over P_2O_5 (Connell, Dixon & Hanes, 1955).

Cell hydrolysates labelled with ³⁵S and containing 100 μg. amino-N, and the ¹⁴C-labelled neutral + acidic amino acid fraction containing 70 μg. amino-N, were applied to Whatman no. 1 paper (27 in. × 23 in.), and methionine converted to the sulphoxide by the peroxide oxidation method of Thompson & Morris (1959), except that 4 drops of 30 % (w/v) hydrogen peroxide were used in a 4 in. watch-glass and the paper exposed for 40 min. Two-dimensional descending paper chro-matography was then used with n-butanol + glacial acetic acid + de-ionized water (60 + 15 + 25, by vol.) as the first solvent run across the grain, and phenol + de-ionized water (4 + 1, v/v) as the second solvent (Smith, 1958). The leading edge of the paper for the run in the first dimension was cut to form 1 cm. serrations and the solvent run for 48–64 hr. The second solvent was run until it almost reached the edge (24–30 hr.). For the separation of lysine, 0·2 ml. of the ¹⁴C-basic fraction containing 6–7 μg. amino-N was applied as a strip 5 cm. long along the base line of Whatman no. 1 paper (18 in. × 9 in. with the leading edge serrated), and this was then developed along the grain in n-butanol + pyridine + de-ionized water (1 + 1 + 1, by vol.) for 58–72 hr. (Morrison, 1953).

Assay of methionine and lysine eluted from chromatograms. The exact position of the amino acids on the chromatograms was defined by radioautography or by using detection ninhydrin spray (Connell *et al.* 1955). The located spots and an adjacent equal area free from amino acids were cut out and suspended from nichrome hooks

in a vacuum desiccator. The papers were flooded with 0·23 ml. borate buffer in methanol (Connell *et al.* 1955) and dried over P_2O_5 *in vacuo*, thus removing ammonia. The dried papers were cut into 0·5 cm. squares and eluted with 1·0 ml. de-ionized water. Standard quantities of lysine and methionine were chromatographed and eluted in the same way. The amino acid content of the eluates was then determined by the method of Moore & Stein (1954).

Measurement of radioactivity. Samples of amino acid eluates described above were transferred quantitatively to 2 cm.^{2} brass planchets for radioactivity counts at infinite thinness. The planchets were prepared with a five degree inward slope on the floor of the well to offset surface tension effects at the periphery during drying. The sample (0·1 ml.) was carefully mixed on the planchet with 5 drops of an ethanolic suspension of purified asbestos powder as absorbent (Francis, Mulligan & Wormall, 1954) and dried *in vacuo* over P_2O_5. The dried samples were counted with a thin mica end-window Geiger-Müller tube and Ekco scaler Type no. 529 A. Under these conditions a ^{14}C-source of activity $2·5 \times 10^{-3}$ μc. produced 610 counts/min. $\pm 1·5$ %. Count rates of replicate planchets agreed within 5 %. Counts of duplicate planchets were taken for 10 min.

RESULTS

Growth responses on solid medium

Medium C agar seeded with a 5 % (v/v) inoculum of the standard washed suspension described in Methods produced the best demonstration of the double requirement for lysine + methionine and was used for all growth tests in solid medium. A typical response after using this inoculum and incubating for 24 hr. at 37° is shown in Pl. 1, fig. 1. A smaller inoculum required prolonged incubation to demonstrate the response, whilst a larger inoculum produced too much background growth. In the presence of lysine the mutant responded to methionine, homocysteine, cystathionine; there was no response to homoserine, homoserine + cystine, threonine, DL-α-aminobutyric acid, L-djenkolic acid or aspartic acid. In the presence of methionine the mutant responded to lysine and to diaminopimelic acid, but there was no response to lanthionine or to DL-allo-δ-(OH)-lysine.

There was no response by the mutant in minimal medium to p-aminobenzoic acid, p-hydroxybenzoic acid, ascorbic acid, biotin, folic acid, glutathione, D-pantothenic acid, pyridoxine, pyridoxal hydrochloride, nicotinic acid, thiamine hydrochloride or cobalamin. Yeast extract produced a good response when a 0·1 % (w/v) solution was applied in a 'fish-spine' bead.

Growth responses in liquid medium

The density of the inoculum had a marked effect on the type of growth response obtained in liquid medium (Table 1). With a heavy inoculum, growth occurred with lysine or methionine or both together, and sometimes in the absence of these supplements; with a dilute inoculum no growth occurred even in the presence of both amino acids. A 1/5 or 1/10 dilution of the standard washed suspension showed the double requirement. When, however, the sole supplement was Difco yeast extract (0·2 mg./ml.) growth occurred even with high dilutions of the inoculum. Acid hydrolysis did not destroy the activity of yeast extract.

Table 1. *Growth responses of* Escherichia coli *15 with different dilutions of a standard suspension as inoculum*

Each 6 in. × ¾ in. tube contained 4·0 ml. medium C supplemented as shown, the inoculum comprising 10 % (v/v) of the final culture volume. The tubes were incubated for 24 hr. at 37°. Tests for viability: all tubes positive. Tests for reversions: all tubes negative. Hydrolysed yeast extract prepared by treating 10 mg. Difco yeast extract with 6 N-HCl at 110° for 16 hr. under nitrogen. After drying over NaOH the hydrolysate was dissolved in water and the culture medium supplemented so that each ml. medium contained hydrolysate from 0·25 mg. yeast extract.

Supplements (10⁻³ M)	Dilutions of the standard suspension used as inoculum						
	1/2·5	1/5	1/10	1/20	1/100	1/1000	1/10,000
				Growth response			
None	+/−	−	−	−	−	−	−
Lysine	+ +	+/−	−	−	−	−	−
Methionine	+ +	−	−	−	−	−	−
Lysine + methionine	+ +	+ +	+ +	+/− or + +	−	−	−
Yeast extract (0·2 mg./ml.)	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Hydrolysed yeast extract	+ +	+ +	+ +	+ +	+ +	+ +	+ +

Responses are indicated thus: − = no visible growth; +/− = faint growth; + + = full growth.

Table 2. *Determination of the limiting amounts of lysine, diaminopimelic acid, and methionine required to support the growth of* Escherichia coli *15*

Experimental conditions as for Table 1 except that the inoculum throughout was 1/5 dilution of the standard suspension. Tests for viability: all tubes positive. Tests for reversions: all tubes negative.

Lysine	Diaminopimelic acid	Methionine	Total growth at 24 hr. (duplicates) (as absorption reading at 420 mμ)	
Final concentration (M)				
—	—	—	0	0
10⁻³	—	—	0	0
—	10⁻³	—	0·02	0·86*
—	—	10⁻³	0	0
10⁻⁵	—	8 × 10⁻⁶	0·88	0·90
8 × 10⁻⁶	—	8 × 10⁻⁶	0·07	0·90
6 × 10⁻⁶	—	8 × 10⁻⁶	0·06	0·05
4 × 10⁻⁶	—	8 × 10⁻⁶	0·05	0·04
2 × 10⁻⁶	—	8 × 10⁻⁶	0·04	0·06
10⁻⁶	—	8 × 10⁻⁶	0·05	0·04
10⁻⁵	—	8 × 10⁻⁶	0·88	0·90
10⁻⁵	—	6 × 10⁻⁶	0·88	0·17
10⁻⁵	—	4 × 10⁻⁶	0·07	0·88
10⁻⁵	—	10⁻⁶	0·88	0·04
—	10⁻³	8 × 10⁻⁶	0·86	0·86
—	10⁻⁴	8 × 10⁻⁶	0·86	0·86
—	10⁻⁵	8 × 10⁻⁶	0·06	0·08
—	8 × 10⁻⁶	8 × 10⁻⁶	0·07	0·06

* With 1/5 inoculum dilutions, full growth occasionally developed in tubes supplemented with lysine or diaminopimelic acid only.

Determination of the minimum amounts of lysine and methionine required to produce growth with a 1/5 dilution of standard suspension as inoculum showed that only trace amounts were required (about 10^{-5} M) but the results became erratic in the vicinity of the 'end-point' (Table 2). An 'all or none' phenomenon was observed, there being either full growth or a faint trace of growth; intermediate amounts of growth were never observed. Similar results were obtained when diaminopimelic acid replaced lysine except that the requirement for diaminopimelic acid was higher than that for lysine.

Quantitative investigation of amino acid synthesis by using ^{14}C-label

Cell hydrolysates labelled with ^{14}C were prepared from the parent strain *Escherichia coli* 518 and from the mutant *E. coli* 15, and fractionated as described under Methods. The observation that homoserine did not replace methionine in growth tests suggested an isotopic competition experiment (Abelson, 1954) in which homoserine (0·1 mg./ml.) was included in the culture medium of the mutant. The total yields of amino acids and recoveries from the fractionation procedure for the three hydrolysates are given in Table 3. The wild-type culture produced somewhat better growth than the mutant cultures; after hydrolysis of the organisms, and subsequent ion-exchange treatment, ninhydrin assays of the basic amino acid fractions and of the neutral + acidic amino acid fractions showed that 95–97 % of the amino-N originally present was recovered.

Table 3. *Comparison of growth, amino nitrogen content and recoveries from the fractionation of* ^{14}C-*labelled cell hydrolysates of* Escherichia coli *518 and* E. coli *15*

Washed organisms were grown in 50 ml. medium C containing 25 μc. ^{14}C-glucose (with 8×10^{-6} M lysine + 4×10^{-6} M methionine supplements for *E. coli* 15). After harvesting and hydrolysis of the organisms, the basic amino acids were separated on the ammonium form of Dowex 50 resin. Amino-N was determined by the ninhydrin method.

	E. coli 518 (wild-type)	*E. coli* 15	*E. coli* 15 with additional supplement of homoserine (0·1 mg./ml.)
Total growth			
Absorption at 420 mμ of 1/10 dilution of culture	0·175	0·150	0·150
Amino-N in cell hydrolysates (μg.)	1360	960	1100
Fractionation of hydrolysate			
Amino-N treated (μg.)	500	460	528
Amino-N recovered in (B) fraction* (μg.)	60	62	71·5
Amino-N recovered in (N + A) fraction† (μg.)	426	376	435
Total recovery of amino acids (μg.)	486	438	506·5
% recovery	97	95	96

* (B) fraction represents 'basic amino acids'. † (N + A) fraction represents 'neutral + acidic amino acids'.

Radioautographs were prepared by exposing 'Kodirex No-Screen X-ray film' to the chromatograms of the various fractions for 30 days (Pl. 1, figs. 2–4). Comparison of the radioautographs of the neutral + acidic amino acid fractions of the hydrolysates showed that the amino acid pattern of the mutant (when grown in

Table 4. *Ninhydrin estimation and radioactivity counts of individual* [14]C-*amino acids in cell hydrolysates of* Escherichia coli *518 and of* E. coli *15*

Washed organisms were grown in 50 ml. medium C containing 25 μc. ^{14}C-glucose (with 8×10^{-6}M lysine $+ 4 \times 10^{-6}$M methionine supplements for *E. coli* 15), organisms hydrolysed and the hydrolysates fractionated on ion exchange columns. Basic amino acids (6 to 7 μg. amino-N) were separated by one dimensional, and neutral + acidic amino acids (70 μg. amino-N) by two dimensional, paper chromatography.

	E. coli 518 (wild-type)	*E. coli* 15	*E. coli* 15 with additional supplement of homoserine (0·1 mg./ml.)
Amino-N in cell hydrolysates (μg.)	1360	960	1100
[14]C-lysine			
Lysine amino-N in eluate (μg./100 μg. amino-N of cell hydrolysate)	4·8	5·65	5·8
Amino-N in lysine supplement (μg.)	0	5·6	5·6
Maximum dilution of isotope possible due to lysine supplement (%)	0	10	9
Specific activity (as c.p.m./μg. amino-N)	504	494	452
[14]C-methionine			
Eluate from chromatogram c.p.m.*	792	750	330
[14]C-threonine			
Eluate from chromatogram c.p.m.	1308	1380	330

* c.p.m. represents 'counts per minute' corrected for background.

minimal lysine + methionine) was similar to that of the wild-type, but differed markedly when homoserine was included in the growth medium, there being then a considerable decrease of radioactivity in methionine and threonine. Quantitative elution of methionine and threonine from the chromatogram followed by radioactive counts established this isotopic competition effect (Table 4). The addition of homoserine to the growth medium decreased the ^{14}C-label in methionine by 56 % and in threonine by 76 %. The decrease was less in the case of methionine because of the

Table 5. *Comparison of growth, amino-N content and radioactivity counts of* [35]S-*amino acids in cell hydrolysates of* Escherichia coli *518 and of* E. coli *15*

Washed organisms were grown in 50 ml. medium C containing 15 μc. ^{35}S-sulphate (with 8×10^{-6}M lysine $+ 4 \times 10^{-6}$M methionine supplements for *E. coli* 15). Organisms were harvested, washed and acid hydrolysed. ^{35}S-Amino acids were separated by two-dimensional paper chromatography. Quantity of hydrolysate chromatographed: 100 μg. amino-N.

	E. coli 518 (wild-type)	*E. coli* 15
Absorption reading at 420 mμ of 1/10 dilution of culture	0·195	0·150
Amino-N in cell hydrolysates (μg.)	1750	1140
Amino-N in eluates of ^{35}S-methionine (sulphoxide, μg./100 μg. amino-N of cell hydrolysate)	2·55	2·45
Amino-N in methionine supplement (μg.)	0	2·8
Maximum dilution of isotope possible due to methionine supplement (%)	0	10
Specific activity of ^{35}S-methionine eluate (c.p.m./μg. amino-N)	2700	2620
Radioactivity of ^{35}S-cystine/cysteic acid eluate (c.p.m.)*	1750	1980

* c.p.m. represents 'counts per minute' corrected for background.

contribution of ^{14}C from cysteine and the β-carbon of serine. Lysine was examined for amino-N content and radioactivity from one-dimensional chromatograms of the basic amino acid fractions. Again it was found that the mutant and the wild-type organisms produced similar amounts of ^{14}C-lysine, but no isotopic competition with homoserine was observed (Table 4).

Quantitative investigation of amino acid synthesis by using ^{35}S-label

Hydrolysates of *Escherichia coli* 518 and *E. coli* 15 labelled with ^{35}S supplied as ^{35}S-sulphate were prepared, and the amino acids separated by two-dimensional paper chromatography. Because of the comparatively high specific activity of ^{35}S-methionine, sufficient material was available in the eluate from each chromatogram to permit measurement of both radioactivity and amino-N; the radioactivity of ^{35}S-cysteine was also measured. The results confirmed that the sulphur amino acid patterns of the mutant (grown in minimal lysine + methionine) and of the wild-type organisms were similar (Table 5).

DISCUSSION

Growth tests on solid medium showed a clear-cut response by *Escherichia coli* 15 to methionine + lysine, and one would therefore expect that the mutant had lost the ability to synthesize these amino acids. Replacement studies indicated that the methionine pathway was blocked at the condensation of homoserine and cysteine to form cystathionine and that the block in lysine biosynthesis occurred somewhere between aspartic acid and diaminopimelic acid. Quantitative studies of the growth of the organism in liquid medium, however, revealed that the situation was complex and that methionine and lysine produced growth in an indirect manner. This is illustrated by the following facts. First, the growth response to methionine + lysine was obtained only with a large inoculum (10^6 organisms/ml.) irrespective of whether substrate amounts (10^{-3}M) or trace amounts (10^{-5}M) of the amino acids were used. Secondly, when yeast extract (0·2 mg./ml.) was the only supplement, the large inoculum was no longer required, a fact which indicated that the growth was not solely due to traces of methionine and lysine which may have been present in the yeast extract. Thirdly, the isotopic evidence showed that methionine and lysine were synthesized *de novo* by the mutant when trigger amounts of supplements were used with an inoculum of 10^6 organisms/ml. The pronounced isotopic competition effect of unlabelled homoserine on the labelling of methionine and threonine indicated that these amino acids were synthesized by the accepted pathways (Fig. 1). The amounts of methionine and lysine found in the cell hydrolysates are in accord with those reported by other investigators (Roberts *et al.* 1955; Anderson *et al.* 1958). As methionine and lysine are stable metabolic end-products in *E. coli* (Abelson *et al.* 1953; Siddiqi, Kozloff, Putnam & Evans, 1952), it follows that there was a true net synthesis and that exogenous methionine and lysine were incorporated unchanged.

It follows from these results that methionine and lysine in the medium were not functioning simply as cellular building materials but rather that they supplied a mechanism to overcome a metabolic block. The large inoculum required suggests that the organisms supplied a necessary component in this system. This requirement was spared by the incorporation of 0·2 mg./ml. yeast extract in the medium. The

active principle in yeast extract does not appear to be an enzyme or a peptide, as its effect was unimpaired when tested subsequent to acid hydrolysis. If enzyme induction or repression is involved in the above system, then lysine and methionine *per se* would not appear to participate, because essentially identical growth responses occurred when these supplements were present from 10^{-3}M to less than 10^{-5}M.

Although the available data do not provide an explanation of all the observed effects, they do suggest that a common link exists in the biosynthetic pathways leading to methionine and lysine. Such a link does not appear to be a common intermediate in view of the separation of the block in the methionine pathway from that of the lysine pathway. It is postulated that the link is a cofactor common to both pathways. The observation that the mutant did not respond to substrate amounts of lysine + methionine at less than 10^6 organisms/ml. may mean that the cofactor participated also in some other undefined aspect of growth. It is hoped that a study of the growth components of the inoculum and of yeast extract will confirm the existence of a common link or cofactor and reveal both the nature of the metabolic block in *Escherichia coli* 15 and the mechanism developed to overcome this block.
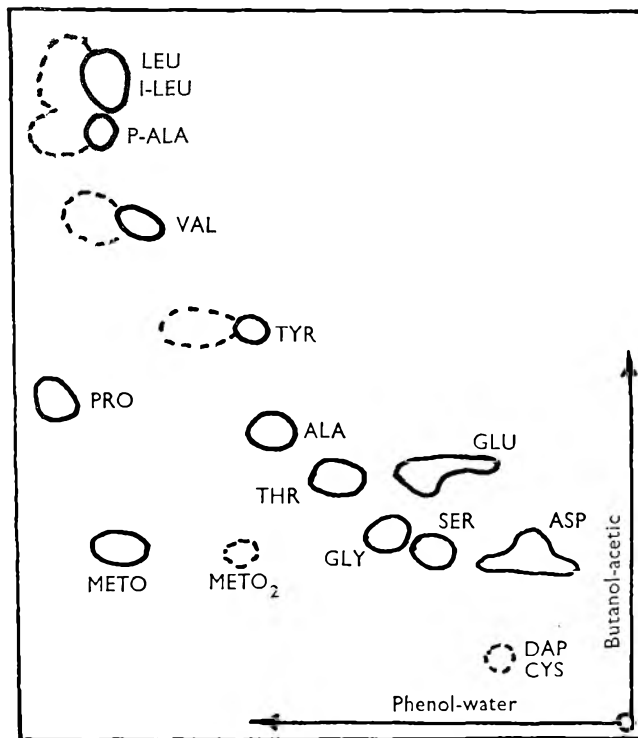
Fig. 2. Chromatographic map showing the location of amino acids on the radioautographs illustrated in Pl. 1, figs. 2–4. The original chromatogram measured 27 in. × 23 in. Abbreviations: LEU = leucine; I-LEU = isoleucine; P-ALA = phenylalanine; VAL = valine; PRO = proline; TYR = tyrosine; ALA = alanine; THR = threonine; GLU = glutamic acid, METO = methionine sulphoxide; METO$_2$ = methionine sulphone; GLY = glycine; SER = serine; ASP = aspartic acid; DAP = diaminopimelic acid; CYS = cystine.

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EXPLANATION OF PLATE 1

Fig. 1. Growth response of *Escherichia coli* 15 in medium C agar. Supplements (10^{-2} M) applied in 'fish-spine' beads and the plate was incubated at 37° for 24 hr. L = lysine; M = methionine.

Fig. 2. Radioautograph of the ^{14}C-labelled neutral + acidic amino acid fraction of the hydrolysed cells after two-dimensional chromatography. The hydrolysate was derived from *Escherichia coli* 518 (wild-type) grown in the presence of ^{14}C-glucose. The amino acids may be identified by referring to Fig. 2, p. 9.

Fig. 3. Radioautograph obtained as in fig. 2, except that the hydrolysate was derived from *E. coli* 15 (mutant) grown in the presence of ^{14}C-glucose and minimal lysine and methionine.

Fig. 4. Radioautograph obtained as in fig. 2, except that the hydrolysate was derived from *E. coli* 15 (mutant) grown in the presence of ^{14}C-glucose and minimal lysine and methionine + homoserine (0·1 mg./ml.). Note the decrease in radioactivity of the methionine (sulphoxide) and threonine spots.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

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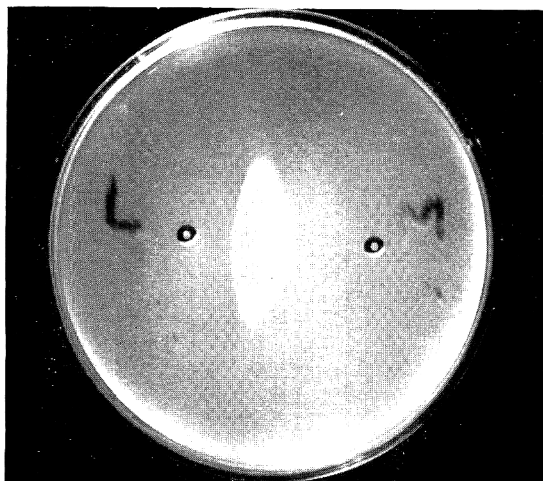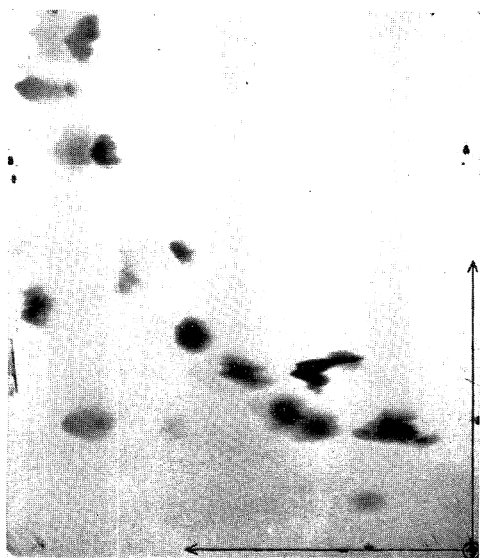
A Defined Medium for *Haemophilus influenzae* and *Haemophilus parainfluenzae*

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SUMMARY

A defined medium is described for the growth of *Haemophilus influenzae* and *H. parainfluenzae* which can be used either as a liquid medium or solidified with agar. The purine and pyrimidine, vitamin, amino acid and mineral salt requirements for three strains have been analysed.

INTRODUCTION

Although a chemically defined medium has been described for *Haemophilus parainfluenzae* (Herbst & Snell, 1949; Herbst & Glinos, 1955), no such medium has been available for *H. influenzae*. This has retarded progress in the study of the genetics of transformable haemophilus strains since biochemical markers could not be studied. It was the possible application in genetic studies that prompted the search for a defined medium for *H. influenzae*. Since the start of this investigation Talmadge & Herriott (1960) described a modification of the tissue culture medium M-199 (Morgan, Morton & Parker, 1950) which allowed the growth of *H. influenzae*. It was felt, nevertheless, that further study was needed and that perhaps a simpler defined medium might be devised.

METHODS

Organisms

Haemophilus influenzae strain Rd of Alexander & Leidy (1953), a rough mutant from a wild Pittman type d, and of known transformability; *H. influenzae* strain RdTr, a strain derived from strain Rd by Dr P. Schaeffer, resistant to streptomycin, erythromycin and novobiocin; and *H. parainfluenzae* strain FID, isolated from a human throat by Dr H. E. Alexander; these will be referred to in the text as strains Rd, RdTr and FID. All strains were maintained on slopes of chocolate agar in cotton-wool plugged test tubes kept at 37°.

Stock culture medium

Tryptone (Difco) was dissolved in glass distilled water at 20 g. together with NaCl at 10 g./l. The pH was brought to about 3 with HCl and activated charcoal added to give a 0·2 % (w/v) suspension. The mixture was allowed to stand with occasional shaking for 15 min. and then filtered. The filtrate was adjusted to pH 7·5 and agar (Davis) added to give a 2 % (w/v) solution. The medium was stored at this stage. When required, oxalated horse blood was added to give a 5 % (v/v) mixture to the molten medium cooled to about 80°, and the blood allowed to turn to a chocolate colour, heated further in a water bath when necessary. The medium was then dispensed as slopes or plates.

Solutions used in the development of the defined medium

Haematin solution. A stock solution of haematin (Roche Products Ltd.) was prepared as follows: 50 mg. haematin were mixed with 12·6 ml. 0·5 M-Na_2HPO_4, heated to dissolve, and 86 ml. glass distilled water + 1·6 ml. M-KH_2PO_4 added. The solution was sterilized by autoclaving at 115° for 10–15 min. One ml. of the stock solution was used in 21 ml. of medium, i.e. a final concentration of 2·4 mg. haematin/100 ml. medium.

Coenzyme 1 solution. Diphosphopyridine nucleotide (Sigma Chemical Co.) was dissolved in glass distilled water to 1 mg./ml., 0·06 ml. of this solution was used in 21 ml. medium, i.e., a final concentration of 0·3 mg. DPN/100 ml.

Glucose-salts solution 1. The solution consisted of 5·0 g. glucose; 17·5 g. K_2HPO_4; 5·0 g. KH_2PO_4; 1·056 g. Na_3 citrate.$5H_2O$; 0·25 g. $MgSO_4.7H_2O$; 2·5 g. $(NH_4)_2SO_4$ in 100 ml. glass distilled water. The mixture was sterilized by autoclaving at 115° for 10 min., and 0·8 ml./21 ml. medium was used.

Glucose-salts solution 2. The solution consisted of 20 g. glucose; 17·5 g. K_2HPO_4; 5·0 g. KH_2PO_4; 0·105 g. Na acetate; 0·25 g. $MgSO_4.7H_2O$ 0·5 g. $Ca(NO_3)_2$; 0·006 g. $FeSO_4.7H_2O$; 0·625 g. $NaHCO_3$ in 100 ml. glass distilled water. The mixture was sterilized by autoclaving at 115° for 10 min.; 0·4 ml. was used per 21 ml. medium.

Yeast extract solution. Yeast extract (Difco) was dissolved in glass distilled water to make a 10 % (w/v) solution, and sterilized by Seitz filtration.

Zeo-carb treated yeast extract. Yeast extract solution (10 % (w/v)) was treated with Zeo-carb 225/H in the proportion of 1 g. resin/3 ml. solution for 20–25 min. with occasional shaking, the mixture then centrifuged, and the supernatant fluid removed and sterilized by Seitz filtration.

Yeast extract ash solution. Yeast extract was ignited repeatedly to constant weight in a platinum dish. The ash was dissolved in concentrated nitric acid, glass distilled water added and the solution adjusted to pH 6·5. Further distilled water was added to give a concentration equivalent to 10 % (w/v) of the initial weight of yeast extract before ashing. The solution was sterilized by autoclaving at 115° for 10 min.; 0·5 ml. of this solution was used in 21 ml. medium.

Agar. New Zealand agar (Davis) at 3 % (w/v) in glass distilled water was sterilized by autoclaving. The molten agar was mixed with the other medium constituents in 25 ml. screw-capped bottles and then poured in plates, the final agar concentration being 1 % (w/v).

Evaluation of medium

Suspensions of the organisms were made in normal saline and a loopful spread over a segment of a plate of medium. After incubation at 37° for 24 hr. and 40 hr. a visual assessment was made of the density and homogeneity of growth and of colony size. Assessment of growth in liquid cultures was made by a visual comparison of the turbidities.

RESULTS

It was decided to start from a non-defined medium which supported growth and which might lend itself to separation into its components. The basal solution contained haematin and DPN as indicated above, with 200 mg. crystalline bovine

albumin (Armour Pharmaceutical Co. Ltd.) per 100 ml. medium, and either glucose-salts solution 1 or glucose-salts solution 2. To this mixture were added the other constituents to be studied, followed by the agar. The albumin requirement will be discussed later.

Yeast extract

Strain FID grew well on basal solution + glucose-salts solution 1 containing yeast extract at a final concentration of 0·25 % (w/v) and above, and still gave small colonies with the yeast extract down to 0·05 % (w/v). Strain Rd also grew but not as well, requiring 40 hr. to give colonies. A somewhat more luxuriant growth was obtained with strain FID on the addition of the 19 amino acids shown in column (a) of Table 1, and the growth developed brown pigmentation. Strain Rd also grew better in the presence of the amino acids, forming dense white colonies with a more transparent rim, and causing a white precipitate to appear in the medium. These colonies were found to have a decreased requirement for haematin and were designated Rd1 ; the properties of this variant will be described elsewhere.

Investigation of yeast extract constituents

Diffusibility. When a sterile dialysis sheet (Visking) was placed over the medium containing double strength yeast extract, the sheet covered with an equal volume of basal solution + glucose-salts solution 1 + amino acids, and the surface inoculated, strain Rd did not grow although strain FID gave pigmented colonies. This suggested that the activity given by the yeast extract which allowed growth of strain Rd was not dialysable. This point was not investigated further.

Effect of Zeo-carb treatment. Addition of yeast extract treated once with Zeo-carb to the basal solution + glucose-salts solution 1 at a final concentration equivalent to 0·25 % (w/v) of untreated yeast extract gave decreased growth of strain FID even in the absence of added amino acids; strain RdTr behaved similarly but only when the concentration of the treated yeast extract was doubled. Strain Rd did not grow even in the presence of added amino acids. After the once-treated yeast extract had been autoclaved at 115° for 10 min., it lost the ability to support strain RdTr, and the growth of strain FID was further diminished. The addition of amino acids stimulated strain FID but had no effect on strain RdTr.

Treatment of the yeast extract three times with Zeo-carb followed by Seitz filtration or autoclaving resulted in a still further diminished ability to support strains FID and RdTr.

In summary, treatment of the yeast extract three times with Zeo-carb followed by autoclaving still permitted some growth of strains RdTr and FID in the absence of added amino acids. Strain Rd did not grow after such treatments of the extract. These findings suggested that, given a medium containing a full complement of amino acids and vitamins, the additional essential constituents provided by untreated yeast extract and removed by resin treatment might include purines, pyrimidines and metal ions.

Replacement of yeast extract

The ability of medium containing treated yeast extract + basal solution + glucose-salts solution to support full growth of all three strains was restored by adding to it a solution of the ten vitamins shown in column (*b*) of Table 1, together with the nineteen amino acids and a solution of ash prepared from yeast extract. Later it was found that the Zeo-carb treated yeast extract itself could be replaced by a solution of purines and pyrimidines. Finally, the ash solution could be replaced by a solution of zinc sulphate.

Table 1. *Mixtures of amino acids and of vitamins used as additions to the basal medium*

(a)		(b)	
Amino acid	mg./l.	Vitamin	mg./l.
Glycine	45·7	*p*-Aminobenzoic acid	8·0
DL-Alanine	45·7		
DL-Valine	137·1	Thiamine	8·0
L-Leucine	137·1	Biotin	8·0
DL-Isoleucine	76·2	Ca pantothenate	8·0
DL-Serine	137·1	Choline chloride	8·0
DL-Threonine	68·55	Folic acid	0·8
DL-Aspartic acid	137·1	Inositol	8·0
L-Glutamic acid	228·5	Nicotinic acid	8·0
L-Arginine HCl	45·7	Pyridoxine HCl	8·0
L-Lysine (HCl)₂	137·1	Riboflavin	0·8
L-Cysteine HCl	118·9		
DL-Methionine	68·55		
Glutathione	59·5		
DL-*β*-Phenylalanine	68·55		
L-Tyrosine	76·2		
L-Tryptophan	22·9		
L-Histidine HCl	56·7		
L-Proline	137·1		

Purine and pyrimidine requirements

The purine and pyrimidine requirements were investigated by using the basal solution + glucose-salts solution 2 + amino acids and vitamins indicated above, together with glutamine + putrescine + yeast extract ash. The purines used were adenine, guanine and hypoxanthine, and the pyrimidines thymine, cytosine and uracil. The results obtained are summarized in Table 2, from which it can be seen that the requirements of all three strains were met by the inclusion of only adenine, guanine and uracil. Strain Rd required adenine and uracil, whilst strain FID required guanine, although uracil, and to a lesser extent, adenine, stimulated growth. A detailed analysis of the requirements of strain RdTr was not made, but it grew well on the mixture containing adenine, guanine and uracil. Neither thymine, cytosine nor hypoxanthine was required by any of the three strains.

Table 2. *Purine and pyrimidine requirements of three Haemophilus strains*

Strain	Essential	Stimulatory	Not required
Rd	Adenine, uracil	—	Thymine, cytosine, hypoxanthine, guanine
FID	Guanine	Uracil, adenine	Thymine, cytosine, hypoxanthine
RdTr	Grows well with adenine + guanine + uracil		

Vitamin requirements

Of the ten vitamins listed in column (b) of Table 1, p-aminobenzoic acid, biotin, choline chloride, folic acid, inositol, nicotinic acid and riboflavin were not required by any of the strains. Table 3 gives the results for the remaining vitamins, and also for glutamine and putrescine. The latter substance was used because of the work of Herbst & Snell (1949) in which putrescine was described as a growth factor for *Haemophilus parainfluenzae*. It can be seen from Table 3 that no added vitamin appeared to be essential for strain Rd, although calcium pantothenate and thiamine were stimulatory, the former being replaceable by pyridoxine. Strains RdTr and FID both required pyridoxine, and were stimulated by pantothenate, although this could be replaced by thiamine for strain FID. Putrescine stimulated all three strains and glutamine was stimulatory for strain RdTr. It was observed that, in the presence of added biotin and folic acid, neither of which was required, strain RdTr then showed a requirement for thiamine. The inclusion of pyridoxine, thiamine, calcium pantothenate, putrescine and glutamine therefore met the vitamin requirement of all three strains.

Table 3. *Vitamin requirements of three Haemophilus strains*

Strain	Essential	Stimulatory	Not required
Rd	—	Ca pantothenate, putrescine, (pyridoxine in absence of Ca-pantothenate)	Biotin, folic acid, thiamine, glutamine, pyridoxine
RdTr	Pyridoxine (thiamine in presence of biotin and folic acid)	Ca pantothenate, glutamine, putrescine	Biotin, folic acid
FID	Pyridoxine	Ca pantothenate, putrescine (thiamine in absence of Ca-pantothenate)	Biotin, folic acid, thiamine, glutamine

Mineral salts requirement

The salts finally used are shown in Table 5.

ZnSO$_4$. It was found that the yeast extract ash could be replaced by a suitable concentration of $ZnSO_4$. The element Zn is a known constituent of some enzyme systems. At a concentration of 3·42 mg. $ZnSO_4.7H_2O$/100 ml. medium (0·0001 M) the growth promoting effect was at least as good as that of the yeast extract ash at the concentration previously stated, but at lower concentrations of the zinc salt there was a rapid diminution of growth. This concentration of zinc represents approximately 1 part/2500. Zinc was spectroscopically undetected in the yeast extract ash but this concentration is below the limit of detection by this method in a sodium and potassium matrix.

Na acetate. This compound had no effect on strains Rd, RdTr or FID. It was retained in the complete medium, however, because it was stimulatory to the growth of the variant Rd1 referred to above, which was investigated simultaneously during the development of this medium.

NaHCO$_3$. This was stimulatory to all three strains.

FeSO$_4$. This was somewhat stimulatory to all three strains at 0·114 mg. $FeSO_4.7H_2O$/100 ml. medium (0·000004 M), but was toxic at higher concentrations.

Phosphates. At concentrations lower than 330 mg. K_2HPO_4/100 ml. medium (0·019 M) and 94 mg. KH_2PO_4/100 ml. medium (0·007 M) there was diminished growth.

CaCl₂. When the medium contained crystalline bovine albumin, $CaCl_2$ was not required to be added, but when the albumin was replaced by polyvinyl alcohol, then $CaCl_2$ at a concentration of 6·75 mg./100 ml. medium (0·0006 M) was stimulatory.

MnCl₂. This was not found to be necessary.

In general strains FID and RdTr were more sensitive to higher concentrations of salts than was strain Rd; strain RdTr was the most sensitive.

Amino acid requirements

Of the nineteen amino acids listed in column (*a*) Table 1, glycine, alanine, serine, threonine, methionine, proline and lysine could be omitted without decreased growth. Glutathione and cysteine were interchangeable, but better growth was given with glutathione. Table 4 gives a summary of the amino acid requirements for each strain. It will be noted that strain Rd did not appear to have an essential requirement for any one amino acid, i.e. the omission of any one of the amino acids from the minimal mixture did not abolish growth.

Table 4. *Amino acid requirements of three Haemophilus strains*

Strain	Essential*	Stimulatory	Not required
Rd	—	Tryptophan, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, isoleucine, phenylalanine, valine	Lysine, glutathione or cysteine, methionine, alanine, glycine, proline, threonine, serine
FID	Isoleucine, valine	Tryptophan, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, phenylalanine	As for strain Rd
RdTr	Isoleucine, leucine, tyrosine	Tryptophan, cysteine or glutathione, aspartic acid, valine (histidine, arginine, and phenylalanine not tested with strain RdTr)	Glutamic acid and as for strain Rd

* Where omission of any one amino acid from the minimum mixture stopped growth.

Albumin

It had been observed that the strains used, *Haemophilus parainfluenzae* in particular, did not grow well in broth media unless albumin was also added, and it seemed likely that the albumin was acting as a protective colloid. Consequently, crystalline bovine albumin at a final concentration of 0·19 % (w/v) had been added to the defined medium throughout its development. It was found, however, that this concentration was having an inhibitory effect; by decreasing the concentration a peak of maximum growth was obtained at a final concentration of 0·019 % (w/v), with a sharp decrease in growth at lower concentrations and a more gradual decrease at higher concentrations; the curve obtained is shown in Fig. 1. Such a result would be expected if the albumin indeed acted as an adsorbent, and an alternative was sought to take its place. Polyvinyl alcohol was tried, with some success. The curve obtained by plotting concentrations of polyvinyl alcohol against growth is also shown in Fig. 1, and it can be seen that it has the same form as that for albumin. The slope

of the peak on the high concentration side is steeper and the growth was somewhat less than with albumin. However, at 64 mg. polyvinyl alcohol/100 ml. medium, the inclusion of $CaCl_2$ at 6·7 mg./100 ml. medium gave growth comparable to that on albumin.

The medium containing either albumin or polyvinyl alcohol proved very satisfactory for several weeks, but on repeating the experiment growth suddenly became poor and even failed completely. It is known that fatty acids may cling even to well-washed glassware. For this reason, the effect of adding sodium oleate was investigated. It was found that growth could be restored in this way. In the presence of albumin, good growth was obtained with 1·92 mg. sodium oleate/100 ml. medium or
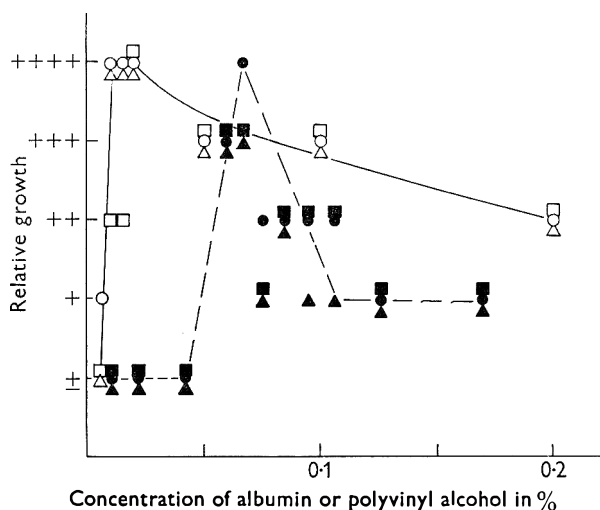
Fig. 1. Effect of crystalline bovine albumin (solid line and open points) and polyvinyl alcohol (broken line and solid points) on the growth of Haemophilus strains—Rd (○, ●), RdTr (△, ▲), and FID (□, ■).

in the presence of polyvinyl alcohol with 0·96 mg./100 ml. medium. Strain Rd was affected only slightly by increase of oleate up to 25-fold with albumin, or to 10-fold with polyvinyl alcohol, but strains FID and RdTr were more sensitive. The latter strains were also more sensitive than strain Rd to lower concentrations of oleate. Oleate in fact completely replaced albumin or polyvinyl alcohol, best growth being obtained at a concentration of 0·48 mg. oleate/100 ml. medium.

Agar

Agar was added to give a final concentration of 1 % (w/v). Unwashed New Zealand (Davis) agar gave the same results when compared with New Zealand agar which had been washed 48 hr. in distilled water, and with Noble (Difco) agar.

The medium finally evolved, therefore, had the composition given in Table 5. The medium was prepared by mixing appropriate volumes of several solutions containing either mixtures of various substances or single components. The table shows the pH of the stock solutions, and whenever possible these were adjusted to about pH 7. Where no pH is indicated, no adjustment was made.

Viable counts (Miles & Misra, 1938) were carried out with suspensions of each

strain, comparing the counts obtained on the defined solid medium containing albumin or polyvinyl alcohol with those obtained on chocolate Tryptone agar. The counts obtained were similar, and showed that the defined media were equal to the chocolate agar in giving growth from inocula of 1 to 10 organisms. Such growth could not consist of selected mutants only. The colonies on the defined medium were somewhat smaller than those obtained on the chocolate tryptone agar and needed incubation for 36 hr. before counting.

Table 5. *Composition of chemically defined medium for certain strains of* Haemophilus influenzae *and* H. parainfluenzae

Except where indicated, constituents were sterilized by autoclaving at 115° for 10–15 min.

Substance	mg./l.	pH of stock solution	Substance	mg./l.	pH of stock solution
DL-Valine	137·1	6·9	Glucose	3810·0	
L-Leucine	137·1	6·9			
DL-Isoleucine	76·2	6·9	K_2HPO_4	3300·0	
DL-Aspartic acid	137·1	6·9	KH_2PO_4	940·0	
L-Glutamic acid	228·5	6·9	$ZnSO_4.7H_2O$	34·2	7·6
L-Arginine HCl	45·7	6·9	Na acetate	41·9	
DL-β-Phenylalanine	68·55	6·9	$NaHCO_3$	225·0	
L-Tyrosine*	76·2	6·9	$MgSO_4.7H_2O$	180·0	
L-Tryptophan*	22·9	6·9	$FeSO_4.7H_2O$*	1·14	
L-Histidine HCl	56·7	6·9	NaCl	8000·0	
L-Cysteine HCl*	118·9				
or					
Glutathione*	59·5		Na oleate	4·8	
Adenine	9·5	6·5	Agar	10,000	
Guanine	0·95	2·0			
Uracil	0·95	6·5			
Pyridoxine HCl*	8·0	7·1			
Thiamine*	8·0	7·1			
Ca pantothenate*	8·0	7·1			
Glutamine*	300·0				
Putrescine*	2·98				
Haematin	23·8				
Coenzyme I*	2·9				

*Sterilized by Seitz filtration.

The defined medium without agar and with albumin allowed growth of all three strains, but only strains Rd and RdTr grew in liquid medium containing polyvinyl alcohol (but no oleate). This suggests that while crystalline bovine albumin or polyvinyl alcohol + $CaCl_2$ are satisfactory alternatives for an agar solidified medium only albumin should be used for liquid medium. The addition of oleate to the albumin-containing medium enhanced growth, particularly that of strains Rd and FID. Oleate without albumin gave somewhat less growth than with albumin.

Five other strains of Haemophilus obtained from the National Collection of Type Cultures (NCTC) were tested for growth on the solid medium. *Haemophilus influenzae* NCTC 4560 and *H. parainfluenzae* NCTC 4101 grew on both the albumin-containing medium and the polyvinyl alcohol-containing medium, whilst *H. canis* NCTC 1659 grew on the albumin-containing medium only. *H. aegyptius* NCTC 8502 and *H. haemolyticus* NCTC 8479 did not grow on either medium. The use of oleate, however

(with or without albumin), allowed growth of the *H. haemolyticus* and also of two isolates of *H. influenzae* recently obtained, one from an eye swab and the other from a specimen of sputum. The *H. aegyptius* strain was not tried on the oleate-containing medium as it was not available at the time of those experiments.

<div align="center">DISCUSSION</div>

These results show that the nutritional difference between *Haemophilus influenzae* and *H. parainfluenzae* is not simply that the former is dependent on added haematin, since differences also occur between the two species in their amino acid, vitamin, purine and pyrimidine requirements, and in their sensitivities to the action of protective colloids. The dependence of *H. influenzae* on haematin, taken together with other cultural characteristics, gives the impression that this species is likely to be more exacting in its nutritional requirements than *H. parainfluenzae*. However, the results were contrary to such a concept, since the growth of strain Rd was not dependent on the presence of any one vitamin or amino acid (in the presence of other vitamin and amino acids), whereas that of strain FID was dependent on the presence of pyridoxine, isoleucine and valine. It is tempting to suggest that the need of strain RdTr for isoleucine, leucine and tyrosine is connected with the resistance of this organism to streptomycin, erythromycin and novobiocin.

The use of crystalline bovine albumin in a defined medium may be objected to. It seems probable that the albumin takes no part as a nutrient and acts only as an adsorbent of growth inhibitors and a substitute for it was sought. It was thought that charcoal would be too active, and indeed Brumfitt (1959) found that *Haemophilus influenzae* was inhibited by 0·5 % (w/v) charcoal although *Bordetella pertussis* grew better in its presence. Starch has been used by several workers (Hornibrook, 1939, 1940; Wilson, 1945; Farrell & Taylor, 1945; Cohen & Wheeler, 1946; Verwey, Thiele, Sage & Schuchardt, 1949) but it was thought that this might hydrolyse to give sugars which would interfere with the use of the medium in a study of the sugar reactions of the group. Talmadge & Herriott (1960) included polyvinyl alcohol in their modified M-199 medium, and it seemed probable that it was used as a detoxifying agent. As the present results showed, polyvinyl alcohol allowed growth comparable to that obtained with albumin (in the presence of low concentrations of Ca ion), but the strains tested were more sensitive to small changes in the concentration of polyvinyl alcohol than they were to similar changes of albumin.

The ability of oleate to replace albumin and polyvinyl alcohol would suggest that oleate also plays a physical rather than a metabolic role. On the other hand, however, the possibility still remains that oleate is required as a metabolite, and it may well be that it performs both metabolic and adsorptive functions. The effective concentration of oleate was about one-twentieth of that of albumin, which might be explained if its micelle is larger than the albumin molecule. When in the presence of albumin, oleate was required at four times the concentration, indicating the expected combination with albumin resulting in loss of activity. It is of interest to note that a loss of activity was also seen in the presence of polyvinyl alcohol.

Comparison of the medium described above with that of Talmadge & Herriott shows several differences. These authors used only seven amino acids but the constituents included biotin, choline chloride and inositol, which were not necessary for the strains used in the present work. Nucleosides, including thymidine, were used by

Talmadge & Herriott, whereas the free purines and pyrimidines were found sufficient in the medium described here and thymine was unnecessary. An interesting difference is that no added zinc ion was needed by the American authors; their medium also included triethanolamine, glycylglycine and Tween 80.

The defined medium described above for Haemophilus should now make it possible to study its metabolism and the genetic control of its metabolic processes.

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Studies on the Growth and Sporulation of Some Species of *Penicillium*

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SUMMARY

The effect of certain carbohydrates, nitrogen compounds and accessory growth factors on the growth and sporulation of several strains of *Penicillium vermiculatum* and *P. wortmanni*, as well as of many commonly occurring non-perithecial species, was studied. An initial low concentration of soluble sugar was conducive to perithecial formation in the two sexual species, and perithecial growth was best with polysaccharides as carbon source. Peptone was the most favourable nitrogen source; asparagine exerted an inhibitory effect in many cases. Of six B-group vitamins tested only thiamine had any marked effect. Similar effects of the nutritional factors were seen with the non-perithecial species generally.

INTRODUCTION

In 1948–49 seven isolations of *Penicillium vermiculatum* and six of *P. wortmanni* were made in this laboratory from rotting jute materials, and were found to display considerable variability in sporulation and other characteristics (Basu, 1951). By 1953, three strains of the first-named were no longer producing perithecia. Physiological studies relating to perithecial formation in *Penicillium* are rare; the present work has been concerned with the effect of nutritional and other factors on the growth and fruiting of *P. vermiculatum* and *P. wortmanni*. A similar but less detailed study has been made also of growth and conidial formation in a number of non-perithecial *Penicillium* species. In the identification of species the classification of Raper & Thom (1949) was followed. Strains of *P. vermiculatum* were given the integral code number *77*; those numbered *77.29*, *77.62* and *77.81* regularly produced perithecia, and *77.80B* did so on malt agar, but only occasionally on potato glucose agar. The six strains of *P. wortmanni* fell into two rather distinct morphological groups (numbered *126* and *130*; Basu, 1951); all fruited normally. Of the non-perithecial species, twenty-nine isolates comprising twenty-two species belonging to the main divisions of the genus were studied; they are enumerated in Table 2. Tests for heterothallism, which was stated by Derx (1926) to be responsible for certain features of fruiting in some strains of *P. luteum*, showed that the species used in this investigation were truly homothallic.

METHODS

All the fungi used here have been subcultured since isolation alternately on malt agar and potato glucose agar at intervals of 6 months. The basal medium used contained: $NaNO_3$, 2 g.; K_2HPO_4, 0·75 g.; KH_2PO_4, 0·25 g.; KCl, 0·5 g.; $MgSO_4$. $7H_2O$, 0·5 g.; water to 1 l.; adjusted to pH 7·0. To the medium was added glucose (unless another carbon source was being investigated) usually to 3 % (w/v). Cultures were ordinarily made in liquid media, of which 10 ml. portions were taken in 50 ml. Pyrex conical flasks and closed with aluminium caps. For solid media (agar 15 g./l.) 20 ml. quantities were taken in 10 cm. Petri dishes. Sterilization was at 115° for 15 min., and incubation at 27°. As a measure of growth mycelial dry weight was estimated; for this purpose the harvested mycelia, after washing with distilled water, and being freed of surplus water with blotting paper, were dried at 105° for 4 hr. and weighed. When cellulose (Whatman filter paper no. 30) was used as carbon source, growth was estimated from the loss in weight of the paper.

RESULTS

Carbon sources

Eight different carbohydrates were tested as carbon source, at 3 % (w/v) in the liquid medium, excepting cellulose which was about 1 % (w/v). The figures in Table 1 show that these carbohydrates differed widely in their capacity to promote growth and to stimulate fruiting of various strains of *Penicillium vermiculatum* and *P. wortmanni*. Maltose was generally the most effective and xylose the least for vegetative growth; but mycelial weight after an arbitrary time does not necessarily give a true measure of the maximum growth attainable. Some strains of *P. vermiculatum* and *P. wortmanni* only attacked cellulose feebly and did not produce perithecia; but two other strains of *P. vermiculatum* which attacked cellulose vigorously also fruited well thereon. Starch and dextrin allowed good vegetative growth of all strains, but encouraged fruiting of *P. wortmanni* only. The contrast between cellulose and starch as substrates for production of strong perithecial growth in these two species is noteworthy.

In similar tests with the non-perithecial species there was a considerable variation in the capacity of the carbohydrates to stimulate sporulation (Table 2). When the medium contained 3 % (w/v) carbohydrate sporulation was often greatest on the polysaccharides and least on glucose; maltose was often superior to other simple sugars. Eight species did not sporulate on cellulose media; all of these were very weakly cellulolytic. Eight strongly cellulolytic species all sporulated very vigorously; but instances were noted in which quite vigorous conidial formation was associated with rather weak cellulolytic capacity, e.g. *Penicillium roseo-purpureum 148* and *P. notatum 138*. There is a fair degree of agreement between the present observations and those of Marsh, Bollenbacher, Butler & Raper (1949) who made a survey of cellulolysis by *Penicillium* species under quite different experimental conditions.

Experiments in which glucose only was supplied in a wide range of concentrations confirmed that for *Penicillium vermiculatum* and *P. wortmanni*, as for many other fungi, fruiting was favoured by a low concentration of soluble carbohydrate in the medium (Table 3). Of the four strains of *P. vermiculatum* tested none formed

Table 1. *Effect of various carbohydrates on growth and fruiting of* Penicillium vermiculatum *and* P. wortmanni

All carbohydrates 3% (w/v) (except cellulose—1%). Intensity of fruiting judged visually, and rated from 0 to 4 (maximum). * = immature perithecia only.

Strain no.	Day of appearance of perithecia								Intensity of fruiting at 14 days								Mycelial dry weight (mg.) at 14 days								Loss of ... (%)
	Xylose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose	Xylose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose	Xylose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose
P. vermiculatum																								
77.29	>14	>14	>14	>14	7	>14	>14	7	0	0	0	0	1*	0	0	3	31	36	53	38	85	82	79	14·
77.62	>14	>14	>14	>14	7	>14	>14	7	0	0	0	0	1*	0	0	4	36	37	54	46	87	83	73	10·
77.80B	>14	>14	>14	>14	>14	>14	>14	9	0	0	0	0	0	0	0	2	35	36	67	41	84	87	80	8·
77.81	>14	>14	>14	>14	7	12	6	14	0	0	0	0	2*	1*	1*	0	27	14	10	24	54	50	47	0·
P. wortmanni																								
126.24	12	12	8	10	8	10	10	>14	3	2	2	2	3	4	3	0	35	46	45	56	94	65	44	23·
126.31	12	11	7	6	8	6	9	>14	2	2	2	2	3	4	4	0	47	51	51	60	99	75	58	2·
126.78	8	8	7	7	8	11	7	>14	2	2	3	3	3	4	4	0	32	36	44	36	89	66	58	2·
130.30	>14	>14	>14	6	5	7	5	>14	0	0	0	2	2	3	4	0	40	39	55	32	74	63	53	0·
130.63	>14	>14	>14	8	5	7	5	>14	0	0	0	2	2	3	4	0	37	38	52	28	79	59	53	0·8
130.64	>14	>14	>14	6	5	7	5	>14	0	0	0	2	2	3	4	0	44	37	61	29	80	75	78	0·

Table 2. Effect of various carbohydrates on the growth and sporulation of various non perithecial Penicillium species

All carbohydrates 3 % concentration (except cellulose, 1 %). Increasing intensity of sporulation denoted by numbers 0 to 4.

Fungus and ref. no.	Intensity of sporulation at 14 days								Mycelial weight at 14 days (mg.)							Loss (%)
	Xylose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose	Xylose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose
Penicillium adametzi 128	1	1	1	1	1	1	2	0	44	27	36	21	72	71	73	0·8
P. canescens series 136	2	2	2	2	3	4	4	3	41	37	43	45	42	50	43	20·5
P. citrinum 37	2	2	2	2	2	3	3	1	42	64	48	60	53	53	66	5·8
P. citrinum 37·1	2	1	2	1	3	4	4	2	72	82	74	64	89	72	71	4·5
P. citrinum 37·2	2	2	3	2	3	4	3	2	56	71	62	50	84	81	74	4·9
P. cyaneum type 124	1	0	1	0	1	1	2	0	75	97	79	100	76	79	84	0·5
P. fellutanum 7	2	2	2	2	2	2	2	1	42	51	51	41	84	86	85	0·7
P. fellutanum 125	0	1	1	2	1	1	1	0	8	27	20	25	20	22	9	3·4
P. fellutanum series 135	2	2	2	3	2	2	4	0	8	50	24	30	55	28	94	1·1
P. funiculosum series 150	2	2	2	2	3	3	4	3	22	32	39	35	22	43	48	36·2
P. implicatum 53	2	2	2	2	2	3	4	1	47	60	66	78	58	73	88	0·6
P. implicatum series 101	0	0	1	0	0	1	1	0	70	98	84	71	110	81	60	3·8
P. luteum 28	0	0	0	0	1	2	1	1	71	75	85	85	98	101	99	0·5
P. notatum 138	2	1	1	0	2	3	3	3	74	70	71	73	79	79	74	5·7
P. ochro-chloron 158·1	0	0	0	0	0	3	3	3	103	120	101	67	106	92	100	37·9
P. oxalicum 96	2	2	3	2	3	3	3	3	66	54	74	58	73	78	105	21·6
P. purpurogenum 134	2	2	2	3	2	4	3	0	35	42	58	52	69	80	51	0
P. roseo-purpureum 148	1	1	1	1	2	3	3	3	78	53	81	50	99	72	69	6·0
P. rubrum 127	0	0	0	0	0	2	2	2	59	59	74	54	67	95	52	11·8
P. simplicissimum 4	1	0	0	0	2	3	4	1	110	120	101	102	111	97	94	0·9
P. steckii 137	1	1	1	1	1	2	2	3	63	48	81	65	83	68	85	13·3
P. tardum 132	1	1	2	1	1	1	1	1	28	52	86	50	41	56	54	0
P. variabile 34	2	2	3	1	2	2	3	3	54	59	55	21	23	91	15	1·7
P. variabile 121	2	0	2	0	2	2	2	1	89	61	82	49	76	96	62	32·0
P. variabile 121·1	0	1	0	1	1	1	2	3	64	32	43	47	52	94	58	38·0
P. variabile 121·2	2	0	2	1	3	2	2	3	73	69	86	53	71	91	68	22·4
P. verruculosum 158	1	1	1	0	2	1	1	3	26	22	28	36	44	35	52	12·9
Penicillium sp. 40	0	0	0	0	0	0	0	0	27	25	36	20	50	52	72	0
Penicillium sp. 98	1	0	0	0	0	3	3	4	58	48	58	37	58	50	68	50·0

perithecia within 14 days on the 1·5 % (w/v) glucose medium. The three *126* strains of *P. wortmanni* were relatively less sensitive to the sugar concentration, but although perithecial frequency was still high at glucose concentrations > 0·5 % (w/v), the perithecia themselves were still immature on the 14th day. In contrast, the three *130* strains of *P. wortmanni* were the most sensitive of all to sugar concentration. Similar experiments with maltose or sucrose gave the same type of result, although on maltose *P. wortmanni 126* strains gave optimum fruiting at a rather higher sugar concentration, namely 2 % (w/v).

Table 3. *Effect of glucose concentration on fruiting of* Penicillium vermiculatum *and* P. wortmanni

Intensity of fruiting judged visually and rated from 0 to 4 (maximum). * = immature perithecia only.

	Day of appearance of perithecia					Intensity of fruiting at 14 days				
	Glucose % (w/v)					Glucose % (w/v)				
	0·1	0·5	1·0	1·5	2·0	0·1	0·5	1·0	1·5	1·0
			P. vermiculatum							
77.29	6	9	> 14	> 14	> 14	3	2*	0	0	0
77.62	7	11	11	> 14	> 14	3	2*	2*	0	0
77.80B	> 14	> 14	> 14	> 14	> 14	0	0	0	0	0
77.81	7	8	> 14	> 14	> 14	2	1	0	0	0
			P. wortmanni							
126.24	6	7	7	7	7	2	2	2*	2*	2*
126.31	6	6	7	6	6	2	3	3*	3*	3*
126.78	7	7	7	7	7	2	3	3*	3*	3*
130.30	6	10	> 14	> 14	> 14	2	1*	0	0	0
130.63	6	10	> 14	> 14	> 14	2	1*	0	0	0
130.64	6	10	> 14	> 14	> 14	2	1*	0	0	0

In a further experiment the amount of sugar actually present in the medium at the onset of fruiting (i.e. when immature perithecia were visible) was determined, with liquid media containing four different initial glucose concentrations. Although exhaustion of sugar did not alone necessarily induce fruiting it seemed that, irrespective of the initial concentration, relatively little glucose ever remained when perithecia appeared. Particularly with the *P. wortmanni 126* strains it was noted that with increasing initial sugar concentrations there was an increasing time-lag between the first appearance of immature perithecia and the final production of ascospores (Table 4).

On agar media, fruiting was always much more rapid and vigorous than in liquid media of the same glucose concentration. Strain *77.80B* of *Penicillium vermiculatum* formed perithecia in glucose concentrations up to 0·5 %; the other three strains of this species produced ascospores at all glucose concentrations tested, as did also the three *130* strains of *P. wortmanni*. The three *126* strains of *P. wortmanni*, which had given only immature perithecia in liquid media, produced ascospores.

Table 4. *The amount of glucose remaining in the medium at onset of fruiting, or after 30 days*

Initial concentration of glucose

Strain no.	0·1 % (10 mg./flask)			0·5 % (50 mg./flask)			1·0 % (100 mg./flask)			2·0 % (200 mg./flask)		
	Day of appearance of perithecia	Day of appearance of ascospores	Total glucose remaining (mg.)	Day of appearance of perithecia	Day of appearance of ascospores	Total glucose remaining (mg.)	Day of appearance of perithecia	Day of appearance of ascospores	Total glucose remaining (mg.)	Day of appearance of perithecia	Day of appearance of ascospores	Total glucose remaining (mg.)
P. vermiculatum												
77.29	7	11	0·16	10	30	0·90	> 30	> 30	0·94	> 30	> 30	2·26
77.62	7	11	0·24	11	18	0·13	22	> 30	1·06	> 30	> 30	2·00
77.80B	> 30	> 30	0·19	> 30	> 30	0·98	> 30	> 30	1·10	> 30	> 30	1·20
77.81	7	11	0·50	8	14	0·48	12	24	1·35	25	> 30	1·55
P. wortmanni												
126.24	7	10	0·59	7	12	1·70	7	16	2·20	7	24	1·35
126.31	7	10	0·50	7	12	0·70	7	16	0·93	7	22	6·10
126.78	7	10	0·40	7	12	0·60	7	17	0·89	7	22	3·00
130.30	6	10	0·21	10	18	0·14	> 30	> 30	0·32	> 30	> 30	1·11
130.63	6	10	0·40	10	18	0·15	> 30	> 30	0·48	> 30	> 30	0·78
130.64	6	10	0·16	> 30	> 30	0·48	> 30	> 30	0·63	> 30	> 30	1·06

Table 5. *Effect of different nitrogen sources on fruiting of Penicillium vermiculatum and P. wortmanni*

Intensity of fruiting judged visually and rated 0 to 4 (maximum). * = immature perithecia.

| | Day of appearance of perithecia | | | | | | | | | | Intensity of fruiting at 14 days | | | | | | | | | |
| | Glucose 0·1 % (w/v) | | | | | Glucose 1·0 % (u/v) | | | | | Glucose 0·1 % (w/v) | | | | | Glucose 1·0 % (w/v) | | | |
Strain no.	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate	Asparagine	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate	Asparagine	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate	Asparagine	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate
P. vermiculatum																			
77.29	6	6	>14	>14	>14	>14	>14	9	>14	>14	2	1*	0	0	0	0	0	4*	0
77.62	6	6	>	>14	>14	>14	>14	9	>14 >	>14	2	1*	0	0	0	0	0	4*	0
77.80B	>14 >14	>14	8	>14	>14	>14	>14	9	9	>14	0	0	1*	0	0	0	0	2*	2
77.81	6	7	5	9	>14	>14	>14	7	10	>14	2	2	3	4*	0	0	0	4	1*
P. wortmanni																			
126.24	5	5	11	>14	>14	8	>14 >	8	8	11	2	2	1	0	0	3	0	4	3
126.31	6	5	11	>14 >	>14	8	>14	9	12	6	2	2	1	0	0	3	0	4	3*
126.78	6	5	10	11	>14	6	6	9	11	6	2	2	1	1	0	3	1*	4	3
130.30	6	5	8	>14	>14	>14	>14	6	6	7	2	2	1	0	0	0	0	3	2
130.63	6	4	8	>14	>14	>14	>14	5	6	7	2	2	1	0	0	0	0	3	2
130.64	6	4	8	>14	>14	>14	>14	6	7	9	1	1*	1*	0	0	0	0	3	1

Table 6. *Effect of different nitrogen sources on the growth and sporulation of various non-perithecial Penicillium species*

Glucose 3% (w/v). Increasing intensity of sporulation denoted by numbers 0 to 4.

Fungus and ref. no.	Intensity of sporulation at 14 days					Mycelial weight at 14 days (mg.)				
	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate	Asparagine	Sodium nitrate	Ammonium sulphate	Peptone	Casein hydrolysate	Asparagine
Penicillium adametzi 128	1	0	0	0	2	33	67	107	83	95
P. canescens series 136	1	1	1	0	1	33	19	30	60	22
P. citrinum 37	2	2	2	3	2	76	50	83	98	90
P. citrinum 37.1	2	2	3	3	2	76	46	72	91	90
P. citrinum 37.2	2	2	3	3	2	79	40	64	92	78
P. cyaneum type 124	0	3	0	0	0	101	62	117	78	133
P. fellutanum 7	3	3	3	3	3	49	82	74	98	62
P. fellutanum 125	1	0	3	1	1	27	31	44	80	31
P. fellutanum series 135	2	0	2	1	1	45	61	56	78	63
P. funiculosum series 150	3	1	2	2	2	38	78	50	71	72
P. implicatum 53	2	2	3	2	2	70	71	59	82	70
P. implicatum series 101	0	0	0	0	0	101	55	204	100	167
P. luteum 28	0	0	2	0	1	79	56	90	95	73
P. notatum 138	2	1	3	2	2	72	43	54	69	82
P. ochro-chloron 153.1	0	0	2	0	1	119	69	143	119	136
P. oxalicum 96	2	1	3	1	2	54	44	71	96	51
P. purpurogenum 134	2	0	2	1	3	46	46	60	60	65
P. roseo-purpureum 148	1	0	3	1	2	48	41	46	77	57
P. rubrum 127	0	0	3	1	1	60	50	101	93	94
P. simplicissimum 4	0	0	2	0	0	124	114	133	135	122
P. steckii 137	1	0	0	1	1	52	44	91	110	75
P. tardum 132	2	2	2	2	2	54	35	75	111	51
P. variabile 34	2	1	1	2	2	61	72	62	105	57
P. variabile 121	0	0	0	0	0	64	30	71	58	53
P. variabile 121.1	1	0	1	1	0	37	28	52	20	38
P. variabile 121.2	0	0	0	0	0	71	28	73	66	63
P. verruculosum 158	2	1	3	3	2	25	39	56	51	40
Penicillium sp. 40	0	0	1	0	0	27	55	143	59	108
Penicillium sp. 98	0	0	4	0	4	43	24	40	14	40

Nitrogen sources

Five nitrogen sources (Na nitrate, ammonium sulphate, peptone, casein acid hydrolysate, asparagine), were tested, all at concentrations equivalent to 0·033 % (w/v) N, in conjunction with glucose (0·1 %, 1·0 %, w/v). At both concentrations of sugar, peptone (Oxo Ltd.) or casein acid hydrolysate ('vitamin-free', University of Bombay) gave the maximum mycelial growth, but this may have been due in part to the fact that these substances increased the amount of available carbon in the medium. It is evident from Tables 5 and 6 that as regards fruiting the picture is complicated. With 0·1 % (w/v) glucose the inorganic sources of nitrogen most often permitted perithecial formation while asparagine (Difco; twice recryst.) and the casein hydrolysate did so but rarely. With 1 % (w/v) glucose organic nitrogen compounds were usually more favourable, the best being peptone. Because these variations in fruiting response emphasized the importance of the concentrations of carbon and nitrogen sources, the effect of different glucose concentrations in a peptone medium was examined (Table 7).

Table 7. *Effect of glucose–peptone balance on fruiting of* Penicillium vermiculatum *and* P. wortmanni

Intensity of fruiting judged visually and rated 0 to 4 (maximum. * = immature perithecia. Peptone concentration throughout equivalent to 0·033 % N.

| | Day of appearance of perithecia | | | | | Intensity of fruiting at 14 days | | | | |
| | Glucose % (w/v) | | | | | Glucose % (w/v) | | | | |
Strain no.	0·1	0·3	0·5	0·7	1·0	0·1	0·3	0·5	0·7	1·0
			P. vermiculatum							
77.29	> 14	8	7	8	10	0	2*	4	4	4*
77.62	> 14	8	6	7	10	0	2*	4	4	4*
77.80B	8	6	8	9	9	1*	3	3	3	1
77.81	7	6	7	7	7	2	3	3	3	3
			P. wortmanni							
126.24	11	10	9	9	8	1	2	3	3	3
126.31	11	9	9	9	8	1	2	3	3	3
126.78	7	6	6	6	7	1	2	3	3	3
130.30	7	6	6	6	7	2	3	3	4	4
130.63	7	6	6	6	7	2	3	3	4	4
130.64	7	6	6	6	7	1	1	2	3	3

With 0·2 % (w/v) peptone (equiv. 0·033 % N), the optimum glucose concentration for fruiting was between 0·3 and 0·7 % (w/v) in most instances for *Penicillium vermiculatum* and *P. wortmanni*. Although a low concentration of sugar was found generally favourable for fruiting of these fungi, *P. vermiculatum* 77.29 and 77.62 did not form perithecia on 0·1 % (w/v) glucose in the presence of peptone; at the same concentration of glucose, 77.80B formed more conidia than (immature) perithecia, but with 1·0 % (w/v) glucose few conidia appeared. The three *130* strains of *P. wortmanni* also formed conidia at the lower glucose concentration. One of the strains of *P. vermiculatum* (77.10) which had become non-perithecial in subculture

produced many conidia on these highly nutritive media, with a maximum on media containing about 20 % (w/v) glucose; no perithecia appeared on media with up to 40 % (w/v) glucose.

Responses to growth factors

Thiamine, riboflavin, nicotinic acid, pyridoxine and Ca pantothenate were added to the 1·0 % (w/v) glucose (nitrate as N source) medium to 0·1 μg. and 0·01 μg./ ml., and biotin to 0·01 μg. and 0·001 μg./ml. (Sterilization at 109° for 5 min. only.) In no case was vegetative growth of *Penicillium vermiculatum* or of *P. wortmanni* appreciably affected, and the only significant effect on sporulation was enhancement of perithecial growth of *P. wortmanni* 130.30 and 130.64 by thiamine, and of 130.64 by nicotinic acid. Experiments in which the concentration of thiamine ranged from 0·001 to 1·0 μg./ml. and the initial pH was between 4·4 and 5·8 showed that the effect of thiamine was much more positive and general at about initial pH 5·4. Mycelial growth was not particularly affected, but fruiting was often accelerated and increased; the three 126 strains of *P. wortmanni* showed acceleration only (Table 8).

Table 8. *Effect of thiamine at different* pH *values on fruiting of* Penicillium vermiculatum *and* P. wortmanni

Intensity of fruiting judged visually and rated 0 to 4 (maximum). * = immature perithecia. Glucose concentration 1·0 % (w/v) and thiamine HCl 0·1 μg./ml., throughout.

Strain no.		Day of appearance of perithecia pH value				Intensity of fruiting at 14 days pH value			
		4·4	4·9	5·4	5·8	4·4	4·9	5·4	5·8
		P. vermiculatum							
77.29	Without thiamine	8	8	10	> 14	1*	2*	1*	0
	With thiamine	8	6	8	> 14	1*	3*	2*	0
77.62	Without thiamine	8	8	10	> 14	1*	2*	1*	0
	With thiamine	8	7	6	> 14	1*	3*	2*	0
77.81	Without thiamine	10	9	11	11	2*	2*	2*	2*
	With thiamine	8	8	7	10	2*	2	3	2*
		P. wortmanii							
126.24	Without thiamine	4	4	6	6	3	3	3	3
	With thiamine	4	4	4	6	3	3	3	3
126.31	Without thiamine	4	4	5	6	3	3	3	3
	With thiamine	4	4	5	6	3	8	3	3
126.78	Without thiamine	4	5	6	6	3	3	3	3
	With thiamine	4	4	4	6	3	3	3	3
130.30	Without thiamine	10	7	9	> 14	1*	1*	1*	0
	With thiamine	10	5	9	11	2*	3	1*	1*
130.63	Without thiamine	11	7	10	> 14	1*	1*	1*	0
	With thiamine	10	5	9	10	2*	3	1*	1*
130.64	Without thiamine	10	6	10	> 14	1*	1*	1*	0
	With thiamine	10	5	9	10	1*	3*	1*	1*

Wide differences in response to the growth factors, in media at pH 7, were seen with the non-perithecial species, as shown in Table 9; in some cases the stimulation of growth was considerable. Effects on sporulation were less marked, but panto-

thenate caused moderate development of conidia in one species (*Penicillium rubrum 127*) which did not sporulate at all on the control medium. Stimulation of vegetative growth and of sporulation did not always coincide. The effect of thiamine was enhanced at initial pH values around 5·0. Mycelial growth was increased in *P. citrinum 37*, *P. fellutanum 7*, *P. implicatum 101*, *P. luteum 28*, *P. roseo-purpureum 148*, and *P. verruculosum 158*; enhanced sporulation was seen in *P. adametzi 128*, *P. canescens* series *136*, *P. fellutanum 7*, *P. steckii 137*, *P. variabile 121.1*, *P. verruculosum 158* and *Penicillium* sp. *98*. Even without thiamine, *P. luteum 28*, *P. rubrum 127*, *P. simplicissimum 4* and *P.* sp. *98*, which gave no conidia in 3 % (w/v) glucose at pH 7, showed mild sporulation at pH 5·0.

Table 9. *Effect of vitamins on growth and sporulation of various non-perithecial* Penicillium *species*

Species and ref. no.	Increased mycelial growth (%) in presence of	Increased sporulation in presence of
P. adametzi 128	Ca pantothenate (35 %)	—
P. canescens series *136*	Nicotinic acid (20 %) biotin (29 %)	—
P. citrinum 37.1	—	Nicotinic acid, riboflavin
P. cyaneum type *124*	Biotin (11 %)	—
P. fellutanum series *135*	Thiamine (37 %), riboflavin (16 %), pyridoxine (33 %)	Thiamine, biotin
P. funiculosum, series *159*	—	Thiamine, nicotinic acid, riboflavin, pyridoxine, Ca pantothenate, biotin
P. notatum 138	—	Thiamine, riboflavin, Ca pantothenate, biotin
P. oxalicum 96	Thiamine (25 %), Ca pantothenate (22 %)	Thiamine, nicotinic acid, riboflavin, pyridoxine, Ca pantothenate
P. purpurogenum 134	Nicotinic acid (36 %), biotin (66 %)	—
P. roseo-purpureum 148	—	Riboflavin, Ca pantothenate
P. rubrum 127	—	Nicotinic acid, Ca pantothenate
P. simplicissimum 4	—	Thiamine, nicotinic acid riboflavin
P. steckii 137	Pyridoxine (13 %), biotin (34 %)	—
P. tardum 132	Riboflavin (11 %), pyridoxine (25 %), Ca pantothenate (14 %), biotin (12 %)	—
P. variabile 121	Pyridoxine (14 %), Ca pantothenate (12 %), biotin (20 %)	Thiamine, nicotinic acid, pyridoxine
P. variabile 121.2	Thiamine (14 %), riboflavin (16 %), pyridoxine (20 %), biotin (20 %)	—
Penicillium sp. *40*	Thiamine (27 %), pyridoxine (37 %), Ca pantothenate (20 %), biotin (20 %)	—

DISCUSSION

Klebs's principle that sexual and asexual sporulation in fungi is generally favoured by exhaustion of nutrients is supported by the results obtained here with various carbohydrates as carbon sources. The polysaccharides markedly stimulated sporulation, possibly because their insolubility did not allow a high concentration of free sugar to accumulate in the medium. However, it is not clear why the strains of *Penicillium vermiculatum* which fruited best on cellulose and gave maximum growth on starch and dextrin did not fruit on starch or dextrin, since these also gave a low concentration of 'sugar' in the medium. The polysaccharides are liable to be contaminated with micronutrients; fungal growth factors have for instance been reported in soluble starch (Cochrane, 1958). The presence of such contaminants may therefore have affected sporulation. It may be significant that thiamine, found by Schopfer (1934) in some samples of maltose prepared from starch, proved to be the most potent of the six growth factors tested here. Fergus (1952), however, found that starch and maltose were utilized but poorly for growth by *P. digitatum*.

Organic nitrogen sources such as peptone are also likely to be contaminated with micronutrients, and another complicating factor is the fact that they may also serve as carbon source. That the two perithecial species gave better and more rapid fruiting with 0·1 % (w/v) glucose when the nitrogen was supplied in the inorganic form may be due to the fact that the organic nitrogen compounds by supplementing glucose as a source of carbon maintained a higher sugar concentration and thus delayed fruiting. In general ammonium-N is believed to be more easily utilized by fungi than nitrate-N. But the results reported here seem to indicate that the reverse is true for growth for the majority of *Penicillium* spp. tested. In no case was the production of conidia better with ammonium sulphate than with sodium nitrate.

Reports of growth factor stimulation of *Penicillium* spp. are rare, but from the striking effects on growth and fruiting shown by natural media such as malt agar and potato-glucose agar it may perhaps be assumed that other still undefined micronutrients are important. Organisms such as *P. implicatum 101* and *P variabile 121*, which sporulated well on defined media when first isolated but which formed conidia sparsely or not at all throughout the present experiments, seem to have developed a requirement for some undefined micronutrients, since they were still able to sporulate on malt agar. The loss of sexual fertility observed in three strains of *P. vermiculatum* can hardly be due to nutritional deficiency since even the use of natural media did not result in production of perithecia.

The seven strains of *Penicillium vermiculatum* can be divided into three groups: *77.29, 77.62* and *77.81* fruited regularly and more or less consistently under ordinary conditions; *77C, 77.10* and *77.32* did not fruit under any conditions in these experiments; *77.80B* produced perithecia only under certain circumstances and then not consistently. This strain may represent a stage in the process of loss of sexual fertility. As regards the six strains of *P. wortmanni* the subgrouping previously proposed by Basu (1951), mainly on morphological and cultural characteristics, appears to be valid also on nutritional grounds; it seems that these should be recognized as different varieties, if not species.

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O-Methylthreonine, a New Bleaching Agent for
Euglena gracilis

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SUMMARY

O-Methylthreonine (OM) inhibits multiplication and photosynthetic pigment
synthesis in *Euglena gracilis* and *Ochromonas danica*; it does not inhibit *Chlamy-
domonas reinhardi* or *Rhodopseudomonas palustris*. Inhibition of multiplication and
pigment synthesis may be prevented by L-isoleucine. OM also causes the permanent
loss of chlorophyll and appreciable loss of carotenoids in *E. gracilis* and this may be
prevented by L-isoleucine, α-aminobutyric acid, α-ketobutyric acid, L-threonine,
L-homoserine, or L-methionine. α-Ketobutyric acid is most effective on a molar
basis and is therefore postulated to be the target of OM inhibition.

Isoleucine plays a role in the biosynthesis of photosynthetic pigments and lipids
in *Euglena gracilis*, for the incorporation of ^{14}C-L-isoleucine radioactivity into both
the pigments and lipids is markedly diminished by bleaching agents which prevent
pigment synthesis without affecting multiplication, i.e. *O*-methylthreonine or strep-
tomycin. When pigment synthesis is blocked amino acids accumulate in the culture
supernate.

INTRODUCTION

Many chlorophyll-containing organisms have been bleached temporarily by an
assortment of chemical and physical agents (Scher & Aaronson, 1958; Aaronson &
Scher, 1960) but only the phytoflagellate, *Euglena gracilis*, has been rendered
permanently apochlorotic—that is, free of chlorophyll and capable of multiplica-
tion—by chemical agents such as streptomycin (Provasoli, Hutner & Schatz, 1948;
Jirovec, 1949) and antihistamines (Gross, Jahn & Bernstein, 1955) and physical
agents such as heat (Pringsheim & Pringsheim, 1952) and ultraviolet irradiation
(Pringsheim, 1958). This phenomenon of apochlorosis is one of the few instances
where a change in the environment results in a specific, permanent, inherited
change in the phenotype of an organism. Other specific environmentally induced and
heritable changes such as bacterial transformation, transduction, or killer factor in
Paramecium, unlike Euglena bleaching, involve the transfer of nucleic acids or
particles such as a bacteriophage or killer factor containing nucleic acids. In
apochlorosis in *E. gracilis* diverse agents operating in unknown ways affect the same
site—the synthesis of chlorophyll, of the plastid or both. We do not know how any of
these agents function to produce apochlorosis.

Recently we were fortunate in obtaining DL-*O*-methylthreonine (OM, also called
β-methoxythreonine) which inhibited multiplication and pigment synthesis of
Euglena gracilis and several metaphyta; this inhibition was annulled by isoleucine

(Gray & Hendlin, 1956). We have confirmed these observations and have shown that OM renders *E. gracilis* permanently apochlorotic and that α-ketobutyrate or amino acids that can be metabolized to α-ketobutyrate prevent bleaching by OM. We also have shown that isoleucine is involved in the synthesis of photosynthetic pigments and lipids.

METHODS

The organisms used were the phytoflagellates *Euglena gracilis* var. *Z*, *Ochromonas danica*, *Chlamydomonas reinhardi*, and the photosynthetic bacterium *Rhodopseudomonas palustris*.

The chemically defined media and the procedures for the growth of these organisms were described elsewhere (Aaronson & Scher, 1960). Multiplication was measured as optical density (OD) with a Welch Densichron (Chicago, Illinois, U.S.A.). Photosynthetic pigments were routinely determined by the extraction of organisms with 3–5·0 ml. portions of methanol; these were pooled and the concentration of pigments assayed by reading the OD with Klett–Summerson colorimeter using the 42 (400–500 mμ) and 66 (640–700 mμ) filters.

Mass culture of organisms permanently bleached by DL-*O*-methylthreonine (OM), streptomycin, or an incubation temperature of 35·5° were grown in chemically defined media at 25° in the light. Pigments were extracted by a variation of the aforementioned method and the supernatant fluids prepared by centrifuging the organisms, filtering the supernatant fluids and concentrating it 10–40-fold by reduced pressure distillation. The concentrated supernatant fluids were examined for porphyrins and nucleic acid derivatives with the Beckman DU spectrophotometer. The supernatant fluids were also examined for nucleic acid derivatives by ascending paper chromatography on Whatman No. 1 paper with a butanol + acetic acid + water (4 + 1 + 5, v/v) solvent system. Amino acids were assayed by adding 1·0 ml. of culture supernatant fluid to a mixture of 1·0 ml. of a solution of 0·5 g. ninhydrin/ 100 ml. acetone, and 3·0 ml. water. This mixture was heated for 5 min. at 90° and the OD read on the Klett–Summerson colorimeter with the 54 (520–580 mμ) filter.

Supernatant fluids were examined for amino acids by both ascending and descending paper chromatography on Whatman No. 1 paper with the following solvent systems: isopropanol + acetic acid + water (7 + 2 + 1, v/v) *n*-butanol + acetic acid + water (12 + 3 + 5, v/v) or methanol + pyridine + water (80 + 4 + 20, v/v). Amino acids were developed by dipping the paper in a solution of 0·2 g. ninhydrin/ 100 ml. acetone, air-drying the paper, and heating 5 min. at 90–100°. Photosynthetic pigments were separated by the method of Strain (1953), with Whatman No. 1 paper soaked in 10 % (v/v) glycerol in methanol, air-drying the paper, and developing pigments in 0·5 g. *n*-propanol in 100 ml. of light petroleum. Pigment spots were identified by their fluorescence or absorption when exposed to Wood's light.

Radioactivity was determined with a Nuclear-Chicago scaler with a model D 47 gas flow Micromil window counter (Nuclear-Chicago, Inc., Chicago 45, Illinois, U.S.A.).

Uniformly-labelled ^{14}C-L-isoleucine was purchased from Schwarz Laboratories, Inc. (Mount Vernon, New York, U.S.A.).

We are indebted to Dr D. Hendlin of the Research Laboratories of Merck Sharp and Dohme, Inc. (Rahway, New Jersey, U.S.A.) for calling our attention to the effect of OM on Euglena and a generous gift of this compound.

The bleached and normal organisms were examined with (i) a Zeiss Lumipan microscope equipped for phase, darkfield, and polarized light microscopy through the kind co-operation of Dr J. J. Lee (Dept. of Micropaleontology, American Museum of Natural History, New York, New York, U.S.A.); (ii) a Zeiss standard microscope GFL equipped for fluorescent microscopy with a Reichert 'Fluorex' maximum-pressure mercury-vapour arc-light source, type HBO 200, through the courtesy of Messrs H. A. Fischer and D. Amsterdam of the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, New York, U.S.A.

RESULTS

Effect of OM on multiplication

OM inhibited multiplication of several photosynthetic flagellates (Tables 1, 2). *Ochromonas danica* was at least ten times more sensitive than *Euglena gracilis*. The photosynthetic bacterium, *Rhodopseudomonas palustris*, and the phytoflagellate, *Chlamydomonas reinhardi*, were not inhibited by OM up to concentrations of 30·0 mg./ 100 ml. The inhibition of multiplication of *O. danica* and *E. gracilis* was annulled by isoleucine; this prevention of inhibition was competitive. The partial inhibition of *E. gracilis* multiplication by OM was also annulled by several other amino acids, aliphatic acids, and certain steroids and their precursors. These were not examined in detail because of the limited supply of OM.

Table 1. *Effect of OM on multiplication and pigment synthesis of* O. danica

Concentration (mg. %, w/v) O-Methylthreonine (OM)	Optical density (as % of normal)					
	No isoleucine			DL-isoleucine 3·0 mg. %, w/v		
	Cell	660 mμ	420 mμ	Cell	660 mμ	420 mμ
0·6	103	86	80	100	107	100
1·0	66	29	23	100	105	100
2·0	20	8	6	103	100	100
4·0	5	—	—	103	82	100

Effect of OM on photosynthetic pigment formation in Euglena gracilis

When grown in OM at concentrations between about 1·0 and 10·0 mg./100 ml., there was no marked inhibition of multiplication but the resulting suspension of organisms was white and the organisms contained little if any carotenoids and no chlorophyll (Table 2). When samples of these organisms were plated on inhibitor-free agar media the resulting colonies were white and successive weekly transfers of the progeny of these colonies (33rd at this writing) in the light remained white, i.e. permanently apochlorotic. Extraction of the photosynthetic pigments of these white organisms revealed no chlorophyll; however, in the absence of OM, carotenoids were synthesized but only 4 % of the normal quantity. Similar low quantities of caro-tenoids were found in dark-grown organisms or organisms bleached by streptomycin or high temperature (35·5°) incubation.

Bleaching of Euglena gracilis

Bleaching by OM was competitively annulled by L-isoleucine, L-threonine, L-homoserine, α-aminobutyric acid or α-ketobutyric acid; α-ketobutyric acid was most

Table 2. *Effect of OM on multiplication and pigment synthesis by* E. gracilis *and its prevention by isoleucine*

Concentration of L-isoleucine (mg. %, w/v)

Concentration (mg. %, w/v) O-methylthreonine	0				1·0				10·0			
	Cell O.D.	Colour of culture	O.D. 660 mμ	O.D. 420 mμ	Cell O.D.	Colour of culture	O.D. 660 mμ	O.D. 420 mμ	Cell O.D.	Colour of culture	O.D. 660 mμ	O.D. 420 mμ
0	1·54	G*	92	333	1·49	G	83	323	1·58	G	88	365
0·1	1·94	G	83	337	1·52	G	70	294	1·46	G	73	299
0·3	2·34	G	47′	222	1·64	G	70	290	1·30	G	00	200
0·6	2·22	W	1	25	2·36	G	53	258	1·26	G	54	242
1·0	1·14	W	1	6	2·58	G	34	177	1·40	G	66	300
3·0	1·54	W	0	0	1·94	W	0	12	1·30	G	45	222
6·0	1·28	W	—	—	1·90	W	0	0	2·12	G	61	320
10·0	1·38	W	—	—	1·48	W	—	—	2·55	W	3	33
30·0	0·02	—	—	—	0·13	W	—	—	1·52	W	0	8
50·0	0	—	—	—	0·02	—	—	—	0·54	W	0	0

* G, Green; W, white.

Table 3. *Compounds preventing the bleaching of* E. gracilis *by*
DL-O-*methylthreonine (OM)*

Compound	Conc. (in mM) needed to prevent bleaching by 0·23 mM of OM

$$OCH_3$$
$$|$$
$$CH_3\text{-}CH\text{-}CH\text{-}COOH$$
$$|$$
$$NH_2$$

DL-*O*-methylthreonine

$$CH_3\text{-}CH_2\text{-}CH\text{-}CH\text{-}COOH$$
$$|\quad|$$
$$CH_3\ NH_2$$

L-isoleucine   0·23

$$OH$$
$$|$$
$$CH_3\text{-}CH\text{-}CH\text{-}COOH$$
$$|$$
$$NH_2$$

L-threonine   0·25

$$CH_2OH\text{-}CH_2\text{-}CH\text{-}COOH$$
$$|$$
$$NH_2$$

L-homoserine   0·50

$$CH_3\text{-}CH_2\text{-}CH\text{-}COOH$$
$$|$$
$$NH_2$$

α-aminobutyric acid   0·29

$$CH_3\text{-}CH_2\text{-}C\text{-}COOH$$
$$\|$$
$$O$$

α-ketobutyric acid   0·1

Table 4. *Effect of isomers of threonine on OM inhibition of Euglena*

Compound concentration (mg. % w/v)	Concentration of OM (mg. % w/v)	
	1·0	3·0
	optical density	
0	2·18 W*	1·42 W*
D-threonine 1·0	2·42 W	1·66 W
3·0	2·62 W	1·94 W
6·0	2·32 W	1·60 W
10·0	2·40 W	1·68 W
L-threonine 1·0	2·12 W	1·28 W
3·0	2·60 G	2·08 W
6·0	2·48 G	2·26 G
10·0	2·54 G	2·58 G

* W, White colour of cell suspension; G, green colour of cell suspension.

effective on a molar basis (Table 3). Bleaching by OM was also annulled by L-methionine and tiglic acid (an intermediate in isoleucine synthesis) and by crotonic acid; these also suppress multiplication at higher concentrations and are therefore not very effective as anti-bleaching agents. The D-isomers of the above amino acids did not prevent the bleaching by OM, e.g. D-threonine (Table 4). They did, however, partially prevent the inhibition of multiplication by OM (Table 5). Mixtures of

Table 5. *Effect of some chemicals on the multiplication and ability to prevent OM bleaching of* E. gracilis

Compound	Highest conc. studied (mg. % w/v or v/v)	Effect on multiplication	Ability to prevent bleaching by OM
	Organic acids		
Acetic acid	100	−	−
3-Acetyl-butyric acid	10	−	−
cis-Aconitic acid	10	−	−
Adipic acid	10	−	−
α-Amino-adipic acid	10	−	−
α-Amino-butyric acid	10	−	+
DL-β-Amino-n-butyric acid	10	−	−
γ-Amino-butyric acid	10	−	−
δ-Amino-levulinic acid	10	−	−
δ-Amino-n-valeric acid	10	−	−
Butyric acid	10	−	−
2-Carboxy-butyric acid	10	−	−
Citric acid	10	−	−
Crotonic acid	10	+(10)	+
2,4-Diamino-butyric acid	10	−	−
DL-Diethylmalate	10	−	−
Dimethylmalonate	100	P (30)	−
Ethyl-2-hydroxy-butyric acid	10	−	−
Ethyl-lactate	10	−	−
Fumaric acid	10	−	−
3-Hydroxy-butyric acid	10	−	−
2-Hydroxy-isobutyric acid	10	−	−
α-Hydroxy-α-methyl-butyric acid	10	−	−
β-Hydroxy-β-methyl-glutaric acid	30	−	−
3-Hydroxypropionic acid	10	−	−
Isobutyric acid	10	−	−
L-Isocitric acid	10	−	−
Isovaleric acid	10	−	−
α-Keto-n-butyric acid	10	−	+
α-Ketoglutaric acid	10	−	−
β-Ketoglutaric acid	10	−	−
β-Ketoglutaric acid, dimethyl ester	10	−	−
α-Keto-isovaleric acid	100	−	−
Lactic acid	10	−	−
Malic acid	10	−	−
3-Methoxybutyric acid	100	P (100)	−
DL-α-Methyl-n-butyric acid	10	−	−
β-Methyl-glutaric acid	10	−	−
Methylmalonic acid	30	−	−
α-Methyl-n-valeric acid	10	−	−
β-Methyl-n-valeric acid	10	−	−
γ-Methyl-n-valeric acid	50	−	−
Oxalacetic acid	10	−	−
Propionic acid	100	−	−
Succinic acid	10	−	−
Tiglic acid	10	+(10)	+

Table 5 (*cont.*)

Compound	Highest conc. studied (mg. % w/v or v/v)	Effect on multiplication	Ability to prevent bleaching by OM
	Amino acids		
D-Alloisoleucine	165	—	—
DL-Allothreonine	10	—	—
Asparagine	10	—	—
Aspartic acid	10	—	—
L-Cysteine	10	—	—
L-Glutamic acid	50	—	—
L-Glutamine	50	—	—
Glycine	100	P (100)	—
L-Homoserine	10	—	+
L-Isoleucine	110	P (66)	+
D-Isoleucine	10	—	+
L-Leucine	110	P (110)	—
D-Leucine	110	P (66)	—
DL-Methionine	10	P (10)	+
β-Methylaspartic acid	10	—	—
O-Methylthreonine	50	+(30)	
DL-Norleucine	330	P (11)	—
DL-Norvaline	150	P (20)	—
DL-Serine	10	—	—
L-Threonine	100	P (30)	+
D-Threonine	100	P (30)	—
L-Valine	100	—	—
D-Valine	100	—	—
	Alcohols and amines		
1-Amino-2-propanol	10	—	—
3-Amino-propanol	10	—	—
2-Amino-2-methyl-propanol	10	—	—
Cyclopentylamine	100	—	
β-Mercaptoethanol	100	+(10)	
2-Methyl-butylamine	10	—	—
Pyridoxamine	10	—	—
Threamine	10	—	—
	Sterols and sterol precursors		
Cholesterol	10	—	—
Ergosterol	10	—	—
Farnesol	10	—	—
Geraniol	10	—	—
Isoprene	10	—	—
Lanosterol	10	—	—
Mevalonic acid lactone	10	—	—
β-Sitosterol	10	—	—
Squalene	10	—	—

* +(10) conc. in mg. which cause complete inhibition. P (10) conc. in mg.% which causes partial inhibition.

amino acids, water-soluble vitamins, NH_4Cl, succinic acid and glycine, alone or together did not prevent the bleaching by OM.

The supernatant fluids of cultures bleached by OM, streptomycin or heat contained large quantities of amino acids. Neither these nor the supernatant fluids from normal cultures grown in the light or in the dark and concentrated 10 times prevented bleaching by OM when used in the range 0·01–10·0 % (v/v). These same supernatant

fluids in the above concentration range as well as the amino acids and keto acids which prevented OM-bleaching failed to re-green OM-bleached organisms.

We examined the bleaching ability of several compounds that resembled the branched-chain amino acids as well as compounds reported to inhibit isoleucine synthesis, e.g. penicillamine inhibition of *Escherichia coli* is prevented by isoleucine, valine or leucine (Aposhian, Blair, Morris & Smithson, 1959). Mercaptoethanol was included because Brachet (1958) reported that it decreased the size of algal chloroplasts. None of these compounds prevented bleaching by OM nor did they act as bleaching agents themselves; some inhibited multiplication (Table 5).

Microscopic examination of permanently bleached Euglena gracilis

Organisms from cultures of *Euglena gracilis* permanently bleached by OM, streptomycin, or elevated incubation temperature (35·5°) were compared with normal green organisms by ordinary light, phase, dark field, fluorescent and polarized light microscopy. No differences in number or appearance of intracellular bodies in any of the bleached organisms were noted; the 'eye spot' was present in all. Pringsheim (1941) noted that incubation for extended periods in the dark was necessary for the disappearance of the eye spot in bleached organisms; our cultures were maintained in the light.

The sole difference noted between bleached organisms and normal green ones was the disappearance of the green colour and absence of the red fluorescence of chlorophyll. Some of the colourless bodies seen in the bleached organisms contained oriented internal structures when examined under polarized light.

Table 6. *Effect of OM on cell and pigment synthesis and the accumulation of amino acids*

Cells were grown for this experiment in 100 ml. volumes in 500 ml. screw-top flasks in light.

	Compound (mg./100 ml. water)	Cell O.D.	Colour of culture	O.D. at		Approx. conc. of amino acids in supernate (mg./ml.)
				660 mμ	420 mμ	
	0	2·22	Green	303	1000	0*
OM	1	1·20†	White	6	57	0·008
	10	1·18†	White	5	69	0·02
OM	1 + L-isoleucine 10	2·46	Green	300	1000	0
	10 + L-isoleucine 10	0·92†	White	7	91	0·166

* Cells grown in presence of amino acids incorporate them almost completely.

† The number of cells here represents a lag in multiplication rate rather than true inhibition and would reach full growth if allowed to incubate longer.

Supernatant fluids of bleached Euglena cultures

The supernatant fluids of experimental cultures in which OM had induced bleaching contained large quantities of amino acids. There were no amino acids in the supernatant fluids of cultures grown without OM or not bleached by OM. Supernatant fluids of cultures in which isoleucine had prevented the bleaching by OM and whose organisms remained green contained only small quantities of isoleucine (Table 6). When organisms permanently bleached by OM, streptomycin, or heat were

grown in a chemically defined medium in the absence of bleaching agents and the supernatant fluids concentrated and chromatographed, large quantities of amino acids also were found in the culture fluids; more amino acids appeared in the culture fluids of streptomycin and heat-bleached than in OM-bleached supernatant fluids.

That some of this accumulated amino-acid material reflects lack of isoleucine utilization is shown by the appearance of radioactivity in amino acids other than isoleucine that accumulate when pigment synthesis is blocked (Table 7).

Table 7. *Effect of bleaching agents on the incorporation of ^{14}C-isoleucine*

Concentration (mg./100 ml. water)	% total radioactivity supplied incorporated into	
	Cells	Methanol extract
^{14}C-L-Isoleucine 10·0	94	42*
$^{14}C + O$-Methylthreonine 3·0	90	15
$^{14}C + O$-Methylthreonine 6·0	89	25
$^{14}C + O$-Methylthreonine 10·0	83†	16
$^{14}C + $ Streptomycin 150·0	80	20

* 22 % of total radioactivity in methanol extract appeared in pigments (carotenoids and chlorophyll).

† 11 % of total radioactivity in supernate appeared in amino acids other than isoleucine.

This amino-acid accumulation presumably results from the cessation of protein synthesis in the chloroplast or the cessation of pigment synthesis. To decide which, organisms were grown in uniformly labelled ^{14}C-L-isoleucine, with and without bleaching agents (Table 7). Usually about 45 % of the radioactivity incorporated from isoleucine appeared in the methanol extract containing photosynthetic pigments and other lipids. Bleaching concentrations of agents that bleach, i.e. OM or streptomycin, decreased the incorporation of ^{14}C-isoleucine into the organisms, 12 % for OM and 15 % for streptomycin. Incorporation of ^{14}C-isoleucine into the methanol extract, which we assume to include photosynthetic pigments as well as many lipids, was reduced 62 % by OM and 50 % by streptomycin.

Multiplication and hence protein synthesis continued in the presence of bleaching concentrations of OM or streptomycin and incorporation of ^{14}C-isoleucine into the cells was relatively slightly depressed while there was a 50 % or greater decrease in the incorporation of ^{14}C-isoleucine into the pigments and lipids of bleached organisms (Table 7).

Chromatography of the methanol extract of green cells revealed that about 22 % of the total radioactivity was in chlorophylls and carotenoids.

Where synthesis of chlorophyll is prevented, several workers (Lascelles, 1955; Larsen, 1952) have reported the appearance of porphyrins in the supernatant fluids; none were detected in the supernatant fluids of any of the bleached organisms studied here.

Escherichia coli partially inhibited by streptomycin accumulated quantities of nucleic acid derivatives (Roth, Amos & Davis, 1960), and it has been suggested that streptomycin impaired the permeability of the cell membrane in this organism (Anand & Davis, 1960). Examination of the supernatant fluids of cultures of normal dark, light-grown, and permanently bleached Euglena revealed 9·0 μg./ml. 260 mμ–

absorbing material in light-grown, 11 μg./ml. in dark-grown, 15 μg./ml. in OM-bleached, 15 μg./ml. in streptomycin-bleached, and 13 μg./ml. in heat-bleached Euglena. There was no marked impairment of permeability to nucleic acids or their derivatives in bleached organisms.

DISCUSSION

O-Methylthreonine killing of leaves of bean, pepper, tobacco, wheat, and tomato was prevented by the simultaneous addition of 2–5 times more isoleucine than OM but not of threonine, serine, leucine, norleucine, or valine. Inhibition of growth and chlorophyll formation in *Euglena gracilis* by OM was prevented by isoleucine and partially prevented by leucine and norleucine (Gray & Hendlin, 1956). We have confirmed their results that inhibition of *E. gracilis* by OM was competitively prevented by isoleucine. Similar results were also obtained with *Ochromonas danica*.

Goodwin & Jamikorn (1954) reported a decrease in multiplication and carotenoid synthesis in dark-grown and streptomycin-containing (bleached) *Euglena gracilis* cultures as compared with light-grown cultures. Gross *et al.* (1955) found little chlorophyll in dark-grown and none in streptomycin- or pyribenzamine-bleached cells while the carotenoid content of dark-grown and streptomycin-bleached was about twice that of pyribenzamine-bleached cells; the colourless euglenid *Astasia longa* had no carotenoids or chlorophyll. Some workers have reported no chlorophyll in dark-adapted Euglena; others have reported a small quantity. This discrepancy probably depends on how long the organisms were cultured in the dark and also the amount of light exposure immediately before pigment extraction. Bleaching by OM, as by other agents, results in absence of chlorophyll and decrease in carotenoids.

OM permanently bleached only *Euglena gracilis*, of the several photosynthetic organisms examined by us; this bleaching was prevented by the simultaneous addition of any one of several amino, or keto acids (Tables 3, 5), most effectively by α-ketobutyric acid. We may infer that OM interferes with the utilization of α-ketobutyrate in the synthesis of the photosynthetic pigments and that isoleucine or threonine are on the route to these pigments since both amino acids prevented bleaching by OM (Table 3, Fig. 1). That amino acids, particularly isoleucine, are involved in pigment synthesis may be deduced also from the incorporation of ^{14}C-isoleucine into pigments (Table 7) and inhibition of this incorporation by bleaching.

Other workers noting interrelationships between amino acids and photosynthetic pigments include Huzisige, Terada, Nishimura & Uemura (1957) who found amino acids increasing the chlorophyll content of resting cells of Euglena, and Glover, Goodwin & Lijinsky (1951) who reported that leucine stimulated synthesis of carotenoids in *Phycomyces blakesleeanus*.

OM interferes with isoleucine (or more likely, its metabolic product, α-ketobutyrate) in other kinds of organisms. Rabinovitz, Olson & Greenberg (1955) found that OM blocked the incorporation of isoleucine into the proteins of Ehrlich ascites carcinoma, an inhibition prevented by isoleucine. O-Methyl-allothreonine did not affect isoleucine incorporation into carcinoma protein.

No other amino acid or organic acid studied prevented bleaching by OM; among those tested were several precursors of chlorophyll such as δ-aminolevulinic acid, and succinate alone or with glycine.

To see whether other inhibitors of isoleucine synthesis had bleaching activity for

PROTEIN SYNTHESIS

Not inhibited by OM (except at high concn.)

Iscleucine   Threonine ⟵ Homoserine

α-Aminobutyric ⟶ α-Ketobutyric acid
acid

Inhibited by OM (at low concn.)

?

Temporary inhibition ⟶   ⟵ Permanent inhibition

CAROTENOIDS   CHLOROPHYLL
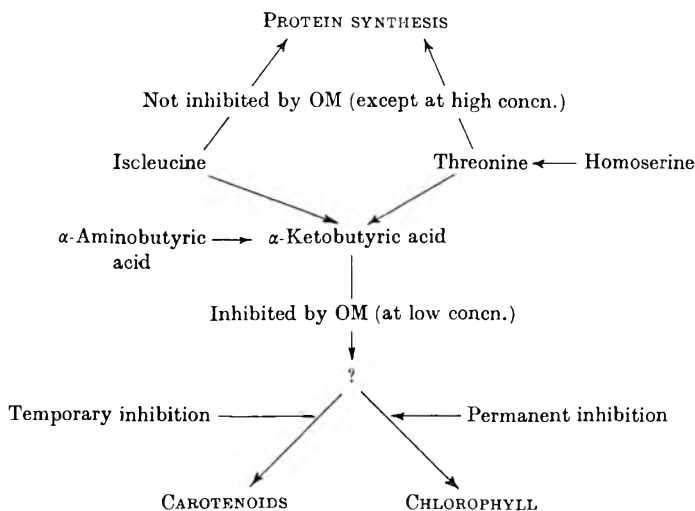
Fig. 1. Suggested sites of inhibition of *Euglena gracilis* by O-methylthreonine (OM).

Euglena gracilis we examined penicillamine which inhibited isoleucine synthesis in *Escherichia coli* and certain substituted cyclopentanes, one of which, cyclopentane glycine, prevented isoleucine synthesis in *Escherichia coli* (Harding & Shive, 1954). None bleached.

Since Euglena does not reproduce sexually, we attempted to bleach two mating strains of *Chlamydomonas reinhardi* to see whether mating a bleached with a green offered information on the primary site of bleaching. Unfortunately Chlamydomonas was not bleached by OM.

Table 8. *Effect of increasing OM on the incidence of bleached individuals in exposed populations*

Compound	Conc. (mg. % w/v)	Colour of culture	No isoleucine		Isoleucine 10·0 mg.	
			White (%)	Green (%)	White (%)	Green (%)
O-Methylthreonine	0·6	White	67	33	—	—
	1·0	White	94	6	—	—
	3·0	White	100	0	—	—
	6·0	White	100	0	—	—
	10·0	White	100	0	67	33
	30·0	White	—	—	100	0
	50·0	White	—	—	100	0

Appearance of colonies from sample of culture when plated†

* No. of viable Euglena cells/ml. varied from *c.* 0·5–3·0 × 10⁶.

† Samples taken from cultures shown in Table 2.

As the concentration of OM increased, the proportion of the Euglena population which showed bleaching increased to 100 % (Table 8). OM selected neither for OM-resistant nor bleached cells, since the growth-rates of bleached and green cultures were identical.

Unlike most externally induced and inherited changes, OM-bleaching of Euglena is a mass phenomenon (Table 8) not clearly dependent on nucleic acid changes as are bacterial transformation, transduction or mutation. Whether this is true of other bleaching agents remains to be seen.

All organisms studied irrespective of bleaching agent have a common feature: they accumulate large amounts of amino acids in the culture fluids, presumably as a consequence of blocked pigment synthesis. The markedly lessened incorporation of ^{14}C-isoleucine into pigment and lipids in OM- and streptomycin-bleached organisms with a much smaller decrease in incorporation into proteins suggests that the amino-acid accumulation resulted from inhibition of the synthesis of plastid pigments and lipids rather than plastid proteins. Anand & Davis (1960) found that normal *Escherichia coli* excreted amino acids; streptomycin treatment caused a fourfold increase in the excretion of amino acids and a greater decrease in intracellular amino acids than in intracellular nucleotides. That isoleucine figures in lipid synthesis may also be inferred from conversion by mouse adipose tissue of isoleucine to fatty acids almost as well as it so converted propionate, acetate, or methylmalonate (Feller & Feist, 1959).

Permanent loss of chlorophyll (apochlorosis or bleaching) as a result of specific environmental changes has been reported by several authors for a few strains of *Euglena gracilis*; it may also occur spontaneously in nature (see review by Pringsheim, 1941 and De Deken-Grenson, 1960). No other photosynthetic organism has been rendered apochlorotic and still able to reproduce. We must note one exception: *Euglena mesnili* kept in the dark lose their chloroplasts permanently (Lwoff & Dusi, 1935).

Provasoli, Hutner & Pintner (1951) claimed the destruction of chloroplasts as well as the inhibition of chlorophyll synthesis. They observed the bleached organisms by fluorescence microscopy which permitted following the fate of the chlorophyll but told nothing of the fate of the plastids. Lwoff & Schaeffer (1949) confirmed bleaching of *Euglena gracilis* by streptomycin but claimed the presence of modified plastids. Vavra (1957) claimed that streptomycin interfered with chloroplast multiplication in *E. gracilis* while De Deken-Grenson & Messen (1958) using light microscopy, said that streptomycin lowered the chloroplast growth-rate so that apochlorotic strains developed as a result of cell multiplication outrunning chloroplast multiplication; this has been withdrawn (De Deken-Grenson & Godts, 1960). Wolken (1956) examined by electron microscopy streptomycin- and heat-treated but no permanently-bleached Euglena; he claimed that both streptomycin and heat destroyed the chloroplast structure but his electron micrographs did not exclude a lamina-free body remaining as a vestige of the chloroplast nor do they depict the fate of the chloroplast in permanently bleached strains. The fate of the colourless chloroplast, if such exists, in apochlorotic cells remains unknown.

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Studies on the Biochemical Basis of the Low Maximum Temperature in a Psychrophilic Cryptococcus

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SUMMARY

A psychrophilic species of *Cryptococcus*, freshly inoculated cultures of which grew well in a glucose + salts + vitamins medium at 16° but not at all at 30°, was induced to grow rapidly for a period at 30° by previous incubation at 16°. A study was made of certain aspects of the biochemical composition of the cryptococcus in cultures maintained at 16° as compared with that in cultures transferred from 16° to 30°, in order to obtain information about the biochemical basis of the low maximum growth temperature for this organism. With the cryptococcus transferred from 16° to 30°, there was rapid utilization of intracellular reserves of total amino acids and total oxo acids. Exogenous sources of amino acids were unable to replenish the intracellular amino acid pool in the cryptococcus incubated at 30°. However, addition of α-oxoglutarate, citrate, or DL-*iso*citrate to cultures transferred to 30° after 120 hr. at 16° enabled the cryptococcus to continue to grow at the higher temperature; stimulation of growth at 30° was most marked in cultures supplemented with α-oxoglutarate. Other tricarboxylic acid cycle and related intermediates (pyruvate, acetate, succinate, fumarate, L-malate, oxaloacetate) had no growth-promoting effect. Stimulation of growth at 30° in the presence of α-oxoglutarate was obtained only in cultures which had been incubated for 2–4 days at 16°, indicating the existence of other temperature-sensitive metabolic processes in addition to those concerned in α-oxoglutarate synthesis.

INTRODUCTION

The existence of psychrophilic strains of micro-organisms has been recognized for many years, but the biochemical basis of the psychrophilic habit is still unknown. Obligate psychrophils are distinguished from mesophils by their ability to grow well at temperatures just above 0° and by their inability to grow at temperatures above about 30°. Both of these characteristics are presumably reflexions of the properties of certain enzymes in these organisms, but little if anything is known about these enzymes. The temperature coefficient of oxidation of several substrates has, however, been shown to be less for psychrophils than for mesophils (Brown, 1957; Ingraham & Bailey, 1959) which suggests that certain of the enzymes in psychrophils are less affected by a decrease in temperature than are the corresponding enzymes in

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mesophils. Attempts have been made to explain the inability of obligate psychrophils to grow at higher temperatures by postulating the existence of exceptionally thermolabile enzymes. Studies on the effect of temperature on the activities of a number of enzymes in cell-free extracts of certain psychrophilic micro-organisms have so far failed to reveal the existence of such thermolabile enzymes (Ingraham & Bailey, 1959).

In a previous paper (Hagen & Rose, 1961) it was reported that a psychrophilic species of the yeast *Cryptococcus* which did not grow in freshly-inoculated cultures at 30° was induced to grow at this temperature when the cultures were incubated initially at 16°. Although this phenomenon was first reported with a newly described species of *Cryptococcus*, it has since been observed with several other psychrophilic yeasts (unpublished observations). This phenomenon appeared to provide a new and more rigorous method for studying the biochemical basis of the low maximum temperature in obligate psychrophils for, by comparing the biochemical activities of yeast in cultures that had been transferred from 16° to 30° with yeast from cultures that had been maintained at 16°, it might be possible to discover differences that would indicate a biochemical basis for the temperature-sensitive habit. The present paper describes experiments which were carried out with the psychrophilic cryptococcus with the object of discovering such differences.

METHODS

Culture methods

Organism. The strain of Cryptococcus used in this study was described by Hagen & Rose (1961). It was maintained on slopes of malt wort agar: 10 %(w/v) spray-dried malt extract ('Muntona', Munton & Fison Ltd., Stowmarket, Suffolk) + 2 % (w/v) agar. Slope cultures were grown at 16° and stored at 3°.

Medium. The basal medium (pH 4·5) used was that described by Rose & Nickerson (1956). The concentration of glucose in this medium was occasionally decreased from 2 to 1 % (w/v); in certain experiments the glucose was replaced by some other carbon source at 0·92 % (w/v). Solutions of various substances were also added to these media as described later; these solutions were adjusted to pH 4·5 and sterilized separately by autoclaving momentarily at 10 lb./sq.in. or by Seitz filtration.

Experimental cultures. Cultures of the cryptococcus were grown in Samco tubes covered with anodized aluminium caps as described by Northam & Norris (1951). Portions of medium (6 ml.) were sterilized and inoculated as described previously (Hagen & Rose, 1961). Growth in the Samco tubes was measured turbidimetrically by a Hilger 'Spekker' absorptiometer (model H. 760) with neutral green-grey filters and a water blank. Turbidity readings were related to the number of cryptococci/ml. by a calibration curve. Large crops of organism for estimations of total intracellular amino acids and oxo acids were obtained by pooling large numbers of these 6 ml. cultures.

Analytical methods

Nucleic acids. The content of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and acid-soluble ultraviolet (u.v.)-absorbing substances in portions (30·0 × 10⁷ organisms) of the cryptococcus which had been washed twice with $M/15$ KH_2PO_4 (pH 4·5) were estimated by the procedures described by Ahmad, Rose & Garg (1961).

Protein in the residue remaining after nucleic acids and related substances had been extracted from the cryptococcus was determined by the micro-Kjeldahl technique (Markham, 1942) with a mercuric oxide catalyst (Miller & Houghton, 1945).

Intracellular amino acid pools. Water-soluble ninhydrin-positive substances were extracted from the cryptococcus by suspending $60 \cdot 0 \times 10^7$ washed organisms in 10 ml. water and holding the suspension for 20 min. in an oil bath at $140°-150°$. On cooling, the supernatant fluid (3–4 ml.) was removed from the cell debris by centrifugation; after being supplemented with washings (2·0 ml.) from the cell debris, the volume of extract was made up to 10·0 ml. with water. These extracts were then assayed for total ninhydrin-positive substances by a modification of the method described by Smith & Agiza (1957) (Mr A. L. S. Munro, unpublished observations). Samples (1·0 ml.) of extract were added to 1·0 ml. citrate buffer (0·05M, pH 5·0) and 1·0 ml. ninhydrin solution (5·50 mg./ml. in citrate buffer) added to each tube. The tubes were then placed in a boiling water bath and 1·0 ml. stannous chloride solution (0·2 %, w/v, in citrate buffer) added to each tube. The tubes were kept in the boiling water bath for 15 min., then removed to a beaker of crushed ice and allowed to cool in the dark. Rapid cooling in the dark was essential in order to obtain consistent results. The contents of each tube were then made up to 10·0 ml. with ice-cold saturated NaCl solution, and this was followed by the addition of 5·0 ml. ice-cold *n*-butanol. Each tube was then stoppered and vigorously shaken, the blue colour being taken up in the butanol layer which was removed, and the optical density of the butanol extract measured at 570 mμ in a Unicam S.P. 600 spectrophotometer in 1 cm. glass cells; blanks were prepared by using 1·0 ml. water instead of 1·0 ml. extract. Optical density readings were related to μg. amino group (NH_2)/ml. by a standard curve prepared by using purified glycine. The results, which are taken as a measure of the intracellular amino acid pool in the cryptococcus, are expressed as μg. $NH_2/60·0 \times 10^7$ organisms.

Intracellular oxo acid pools. Extracts for the determination of total oxo acids in the cryptococcus were prepared in the same way as for the determination of amino acid pools, except that 600×10^7 organisms were used. Recoveries of 98·6–99·2 % were obtained when α-oxoglutaric acid and pyruvic acid were subjected to this extraction procedure. The total oxo acid contents of the extracts (10·0 ml.) were determined by the 2:4-dinitrophenylhydrazine method described by Friedemann & Haugen (1943). Optical density readings were related to the oxo acid content by a standard curve prepared by using purified α-oxoglutaric acid. Results are expressed as μg. α-oxoglutaric acid equivalent/$60·0 \times 10^7$ organisms.

Chromatography. Amino acid extracts (8·0 ml.) from cryptococci were evaporated on a boiling water bath to 0·5 ml., and examined chromatographically on Whatman No. 1 paper. A solvent consisting of *n*-butanol + glacial acetic acid + water (4 + 1 + 5 by vol.) was used, the butanol layer acting as the mobile phase. Extracts were applied on the paper as spots (about 2 μl.), larger amounts being applied by successive application of 2 μl. drops. A measured sample of extract containing 10·0 μg. NH_2 was applied on each spot; controls containing mixtures of known amino acids were run with each chromatogram. Papers were irrigated for 40–44 hr. at room temperature (about 18–20°) and, when the solvent front had descended off the paper, they were removed and dried with a hair dryer. The papers were developed by spraying with a solution of ninhydrin (0·1 %, w/v) in *n*-butanol.

For chromatographic examination of the oxo acids, the sodium carbonate solution of the oxo acid 2:4-dinitrophenylhydrazones obtained during the quantitative estimation of the total oxo acids (Friedemann & Haugen, 1943) was adjusted to about pH 2·0 using conc. HCl, and extracted with ethyl acetate (5·0 ml.). This ethyl acetate extract was then evaporated to dryness in a stream of air at room temperature, and the residue taken up in 0·2 ml. ethyl acetate. This solution was then spotted on Whatman No. 4 paper, and the oxo acid 2:4-dinitrophenylhydrazones separated by descending chromatography using a solvent consisting of n-butanol + water + ethanol (95 %, v/v) (5 + 4 + 1 by vol.), the butanol being used as the mobile phase (Meister & Abendschein, 1956). Papers were irrigated for 10–14 hr. at room temperature and, after drying with a hair dryer, were examined under u.v. radiation; occasionally, they were also sprayed with a solution of NaOH (1·5 N) which caused the oxo acid 2:4-dinitrophenyl hydrazones to appear as brown spots on the paper. Controls of α-oxo-glutaric, oxaloacetic and pyruvic acids were run with each chromatogram.

RESULTS

Preliminary experiments did not reveal any marked differences in the contents of DNA, RNA, acid-soluble ultraviolet-absorbing substances or protein in the cryptococcus grown at 16° compared with cryptococci from cultures of the same age which had been transferred to 30° after 120 hr. at 16°. But, when the amounts of total intracellular amino acids in the cryptococci grown under each of these conditions were compared, then certain major differences were observed.

Effect of change in incubation temperature on the size of the intracellular amino acid pool

The data in Fig. 1 show the effect of varying the incubation temperature between 16° and 30° on growth of, and the size of the intracellular amino acid pool in, the cryptococcus. Transferring cultures to 30° after 72 hr. at 16° accelerated growth of the yeast for approximately 120 hr., after which time there was no further increase in the turbidity of cultures (Hagen & Rose, 1961). This temporary acceleration in the growth rate was accompanied by a rapid decline in the amount of total intracellular amino acids in the cryptococcus as compared with yeast maintained at 16°. When cultures which had been transferred from 16° to 30° were returned to 16° after 144 hr. at the higher temperature, the cryptococcus again started to grow. This second period of incubation at 16° coincided with an increase in the size of the intracellular amino acid pool which, however, was again depleted when these cultures were transferred for a second time to 30° after 144 hr. incubation at 16°.

This fall in the content of intracellular amino acids in cryptococci transferred from 16° to 30° was shown not to be the result of excretion of ninhydrin-positive substances. Portions (10 ml.) of cell-free spent medium from 168 hr. and 216 hr. cultures that had been maintained at 16° or had been transferred from 16° to 30° after 120 hr. were made alkaline with 2 N NaOH and heated for 30 min. in a boiling water bath to liberate ammonia. The solutions were neutralized and concentrated on a boiling water bath to a volume of 2 ml. These concentrates were then examined chromatographically for the presence of ninhydrin-positive substances. Each of the cell-free media showed a faint trace of only one ninhydrin-positive substance which migrated

identically with alanine. There was, however, no detectable difference in the intensity of this spot in chromatograms of concentrates of spent media from cultures that had been maintained at 16° as compared with cultures that had been transferred from 16° to 30°.

Chromatographic examination of concentrates prepared from cell extracts revealed that, when cultures were transferred from 16° to 30°, there was a rapid decline in the amount of glutamic acid in the intracellular amino acid pool; in chromatograms of extracts from cryptococci which had stopped dividing at 30°, the glutamic acid spot was no longer visible. This amino acid reappeared in chromatograms of extracts of the
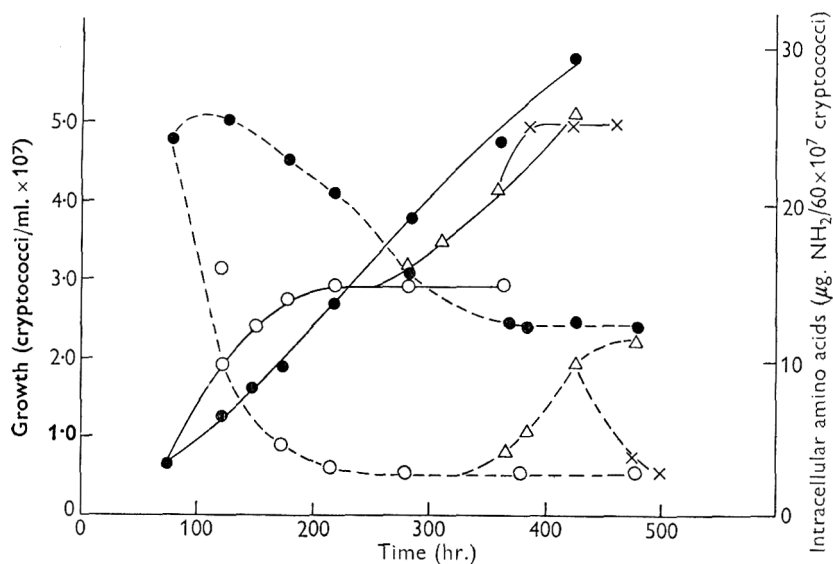
Fig. 1. Growth of cryptococci (———), and size of the intracellular amino acid pool (- - - -), following the transfer of cultures from 16° (●) to 30° (○) after 72 hr., followed by a return to 16° (△) after a further 144 hr. The effect of a second transfer from 16° to 30° is also shown (×).

cryptococcus from cultures that had been returned to 16° after a period at 30°, only to disappear again when the cultures were re-incubated at 30°.

Since these results suggested that the inability of the cryptococcus to continue multiplication at 30°, after being transferred from 16°, might be caused in part by a deficiency in the amino acid synthesizing capacities of the cryptococcus, a study was made of the effect of supplementing cultures with amino acids, in the form of bacteriological peptone (Difco), yeast extract (Difco) or DL-glutamic acid, on growth of, and content of intracellular amino acids in, the cryptococcus. These sources of amino acids were added as solutions (12 %, w/v; pH 4·5) to cultures which had been transferred from 16° to 30° after 144 hr., to give a concentration in the cultures of 0·92 % (w/v). The effect of adding a solution of bacteriological peptone is shown in the data in Table 1. These results show that addition of peptone to cultures of the cryptococcus maintained at 16° stimulated growth to some extent, and also brought about an increase in the size of the intracellular amino acid pool. But addition of peptone did not have any detectable effect on growth of the cryptococcus in cultures

that had been transferred to 30°, while the size of the intracellular amino acid pool in this organism increased only very slightly. Similar results were obtained following additions of yeast extract or glutamic acid to cultures of the cryptococcus.

Table 1. *Effect of addition of peptone on growth of, and content of total intracellular amino acids in the cryptococcus at 16° and 30°*

Cultures (6 ml.) of the cryptococcus, grown in a medium containing 2 % (w/v) glucose, were transferred from 16° to 30° after incubation for 144 hr.; control cultures were maintained at 16°. At the time of transfer, certain of these cultures received 0·5 ml. sterile 12 % (w/v) solution (pH 4·5) of bacteriological peptone. At the same time, certain of the cultures maintained at 16° were similarly supplemented with peptone.

Incubation (hr.)		Growth (cryptococci/ml. × 10⁷)		Intracellular amino acid pool (μg. NH$_2$/60 × 10^7 cryptococci)	
16°	30°	No addition	Peptone added	No addition	Peptone added
144	—	2·20	—	23·5	—
192	—	2·90	3·10	12·5	13·4
144	48	3·30	3·20	7·5	9·8
240	—	3·50	3·80	10·0	15·2
144	96	3·60	3·50	4·0	6·2
288	—	4·10	4·50	8·4	18·1
144	144	3·60	3·50	3·5	6·7

Table 2. *Effect of change in incubation temperature from 16° to 30° on the total oxo acid content of the cryptococcus*

Incubation (hr.)		Growth (cryptococci/ml. × 10⁷)	Total intracellular oxo acids (μg. α-oxoglutaric acid equivalent/ 60 × 10^7 cryptococci)
16°	30°		
144	—	2·17	0·37
96	48	2·55	0·23
168	—	2·50	0·41
120	48	2·79	0·26
192	—	3·06	0·45
96	96	2·65	0·12
216	—	3·20	0·48
120	96	3·10	0·09

Effect of change in incubation temperature on the size of the intracellular oxo acid pool

The results described in the previous section suggested that one reason for the failure of the cryptococcus to continue multiplication at 30° after being transferred from 16° was because it lacked the ability to synthesize amino acids and to accumulate these compounds from the medium. A study was therefore made of the effect of a change of incubation temperature on the total oxo acid content of the cryptococcus, since certain key amino acids are known to be synthesized from α-oxo acids. The results of this study are summarized in Table 2. These data show that, although the total oxo acid content of the cryptococcus increased slightly during incubation at 16°, there was, after an initial slight increase, a steady decline in the total amounts of

these acids in the cryptococcus from cultures that had been transferred from 16° to 30°. Chromatographic examination of extracts from cryptococci which had been maintained at 16° and from cryptococci which had been transferred from 16° to 30° revealed the presence of three oxo acids which ran identically with α-oxoglutarate, oxaloacetate and pyruvate. The pyruvic acid spot always appeared most intense, while that of oxaloacetate was least intense.

Influence of tricarboxylic acid cycle intermediates on growth of the cryptococcus at 30°

The data in Table 2 suggest that, at 30°, the cryptococcus is unable to synthesize adequate amounts of oxo acids. Accordingly, an examination was made of the effect of adding certain oxo acids and tricarboxylic acid cycle intermediates to cultures that had been transferred from 16° to 30° after 120 hr.; additions were made at the time of transfer or after incubation for 72 hr. at the higher temperature. The
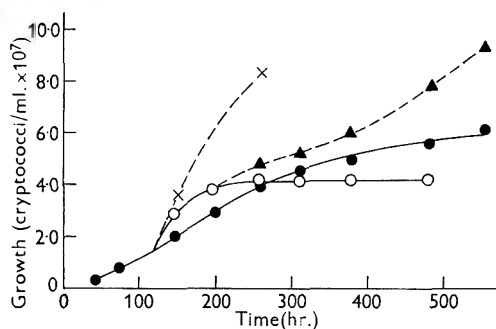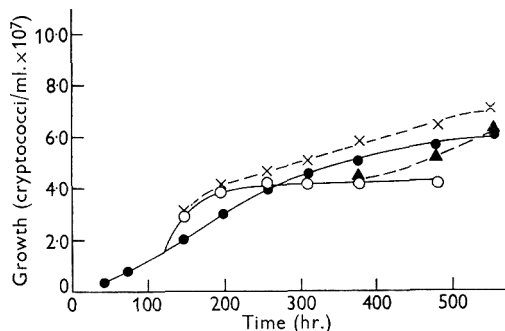
Figs. 2, 3. Effect of addition of α-oxoglutarate (Fig. 2), and citrate (Fig. 3) on growth of the cryptococcus in cultures transferred from 16° to 30° after 120 hr. Cultures (6 ml.) received 0·5 ml. of a sterile 12 % (w/v) solution (pH 4·5) of the potassium salt of the acid on being transferred to 30° (× - - - ×) or after 72 hr. incubation at 30° (▲- - -▲). Control curves show growth of the cryptococcus in cultures maintained at 16° (●—●) and in cultures transferred to 30° after 120 hr. at 16° but which received no additions (○—○). The basal medium contained 1 % (w/v) glucose.

compounds tested were pyruvic, acetic, citric, DL-*iso*citric, α-oxoglutaric, succinic, fumaric, L-malic and oxaloacetic acids. These compounds were added separately to cultures as solutions (12 %, w/v; pH 4·5) of their potassium or sodium salts to give a concentration of 0·92 % (w/v) in the culture. Initially, each compound was tested for its ability to act as the sole carbon source in the medium at a concentration of 0·92 % (w/v). All, with the exception of acetate and *iso*citrate, were capable of supporting growth of the cryptococcus at 16°. However, growth at 30° in cultures transferred from 16° was stimulated only in cultures that had received citric, *iso*citric or α-oxoglutaric acids. Addition of each of the other compounds tested, with the exception of acetate, had no detectable effect on growth of the cryptococcus at 30°; addition of acetate brought about an almost immediate cessation of growth. The stimulatory effect of α-oxoglutarate was most marked when this compound was added at the time of transfer from 16° to 30° (Fig. 2). When the oxo acid was added after incubation for 72 hr. at 30°, there was a lag period before the cryptococcus began to grow

quickly. Addition of citrate (Fig. 3) or isocitrate caused much less stimulation of growth, although there was again a lag period before the cryptococcus grew following addition of these acids to cultures which had been incubated at 30° for 72 hr. There was no change in the pattern of growth when peptone, at a concentration in the culture of 0·92 % (w/v), was added together with citrate or isocitrate. Addition of succinate, fumarate, malate or oxaloacetate, in the presence or absence of peptone, not only failed to stimulate growth of the cryptococcus at 30° but also had no effect on the total oxo acid content of the organism; moreover, there was no change in the
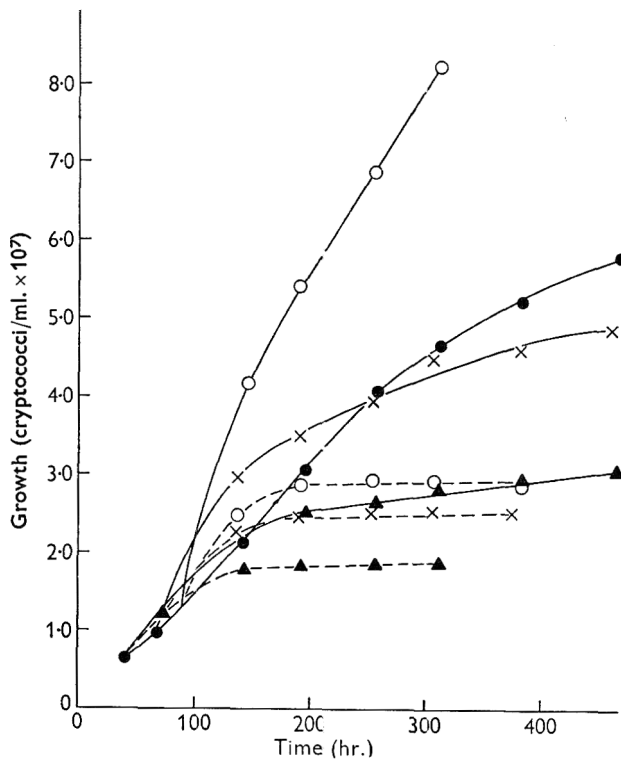
Fig. 4. Effect of addition of α-oxoglutarate on growth of the cryptococcus in cultures transferred from 16° to 30° after 48 hr. (▲—▲), 72 hr. (×—×) and 96 hr. (○—○). At the time of transfer, each of the triplicate cultures received 0·5 ml. of a sterile 12 % (w/v) solution (pH 4·5) of potassium α-oxoglutarate. Growth of control cultures which did not receive α-oxoglutarate on being transferred to 30° is shown with dotted lines. Growth of cultures maintained throughout at 16° is also shown (●—●).

proportions of the three oxo acids, as detected chromatographically, in extracts from cryptococci grown in media containing any one of these tricarboxylic acid cycle intermediates.

Although addition of α-oxoglutarate, at the time of transfer, to cultures which had been incubated at 16° for 120 hr., allowed the cryptococcus to continue to grow at 30°, freshly inoculated cultures containing α-oxoglutarate did not grow when incubated at this temperature. A study of the effect of the time of prior incubation at 16°

on the subsequent growth at 30° in the presence of α-oxoglutarate showed that an incubation period of about 96 hr. at the lower temperature was required before addition of the oxo acid brought about a maximum stimulation of growth at 30° (Fig. 4).

DISCUSSION

The results reported in this paper are concerned with the biochemical basis of only one of the two properties which distinguish obligate psychrophilic micro-organisms from mesophilic species: namely, the inability to grow at temperatures of 30° and above. The data obtained show that, in a psychrophilic species of *Cryptococcus*, this property can be explained, in part, by the inability of the organism to synthesize adequate quantities of α-oxoglutarate. Thus, addition of this oxo acid to cultures that had been transferred to 30° after at least 96 hr. at 16° brought about rapid growth of the cryptococcus at the higher temperature. Inability to synthesize α-oxoglutarate at 30° presumably explains the marked decline in the size of the amino acid pool in cryptococci transferred from 16° to 30° and, in particular, the rapid utilization of the glutamic acid in this pool. It is also possible that this impairment in the metabolic processes leading to synthesis of α-oxoglutarate is accompanied by a derangement in the respiratory metabolism of the cryptococcus. In the present study, however, no attempt was made to measure the respiratory activity of the cryptococcus at various temperatures. But the finding that, at 30°, the cryptococcus was apparently unable to assimilate appreciable quantities of exogenous amino acids, even when these compounds were deficient in the organisms, suggests that, at this temperature, the cryptococcus may be incapable of furnishing the supply of energy needed for the uptake of these nutrients.

From the data available, it is not possible, however, to state precisely the location of the metabolic lesions that are responsible for the increased nutritional demand for α-oxoglutarate at 30°. The results of studies on the uptake of tricarboxylic acid cycle intermediates and related compounds by micro-organisms are frequently not easy to interpret because of the existence of permeability barriers which prevent free access of externally added substrates to intracellular enzyme systems (Barnett & Kornberg, 1960). But, since only two of the intermediates tested (acetate, *iso*citrate) were unable to serve as sole carbon source in the medium, it must be presumed that each of the other compounds tested can enter the cryptococci; it would seem too that *iso*citrate can be assimilated in glucose-containing media. Since both citrate and *iso*citrate are capable of satisfying the increased nutritional demand at 30°, albeit more slowly than α-oxoglutarate, it might be assumed that the metabolic lesions responsible for this demand are in the pathway between glucose and citrate. It is possible that certain of the reactions involved in the conversion of glucose to pyruvate are impaired at 30°. But, since addition of pyruvate to cultures transferred from 16° to 30° does not lead to any increase in growth at the higher temperature, it would seem likely that at least one of the lesions lies among the reactions involved in the conversion of pyruvate to citrate.

Exogenous α-oxoglutarate is presumably oxidized to succinate by the cryptococcus growing at 30°, but, from the data available, it is not possible to state whether the succinate so formed is oxidized further. However, the provision of exogenous sources of succinate and of intermediates formed from succinate (fumarate, malate,

oxaloacetate), in the presence or absence of amino acids, did not enable the crypto-coccus to grow at 30°. This suggests that incubation at this temperature may result in the formation of metabolic lesions among the reactions involved in the transforma-tion of these tricarboxylic acid cycle intermediates. Support for this view comes from the finding that there was no change in the proportions of the three oxo acids in extracts from cryptococci grown in media containing these tricarboxylic acid cycle intermediates.

At least two theories have been put forward to explain the low maximum tem-perature of obligate psychrophilic micro-organisms. One of these postulates the existence of exceptionally thermolabile enzymes in psychrophilic micro-organisms, while the other attempts to explain the inability of these organisms to grow at higher temperatures by assuming that, at these temperatures, the cells synthesize or accumulate a metabolic poison. Since addition of α-oxoglutarate to cultures of the cryptococcus which had been transferred from 16° to 30° brought about an almost immediate increase in the rate of growth at 30°, it seems unlikely that, in this organism, the low maximum temperature is a result of the accumulation of a meta-bolic poison. Further evidence to support this view comes from the finding (un-published observations) that extracts from cryptococci which had been grown at 16° and then transferred to 30° were not capable of inhibiting growth of the organism at 16°. One is led to conclude, therefore, that the temperature-sensitive character of this psychrophil is probably explained by the presence of certain exceptionally thermolabile enzymes. Such enzymes have not been reported in psychrophils to date (Ingraham & Bailey, 1959); they have, however, been found in mutant strains of certain mesophilic micro-organisms (Horowitz & Fling, 1956; Maas & Davis, 1952), and there is also evidence to suggest that certain enzymes in wild-type mesophils may have relatively low temperature maxima (Lichstein & Begue, 1960; Sherman, 1959) and that these enzymes may form the basis of the temperature-sensitive sys-tem that is responsible for the onset of synchronous growth following repeated changes in incubation temperature (Eichel, 1956).

The finding that the addition of α-oxoglutarate, citrate or *iso*citrate to freshly inoculated cultures of the cryptococcus did not enable it to grow at 30° indicates the presence of other abnormally temperature-sensitive metabolic processes in addition to those involved in α-oxoglutarate synthesis. Apparently, however, the thermo-lability of these processes is no longer growth-limiting after the cryptococcus has been incubated for 96 hr. at 16°. It is possible that these other temperature-sensitive processes may be concerned with the synthesis of certain enzymes during the early stages of growth; in this connexion, it is interesting to note that the process of enzyme induction in various micro-organisms has been shown to be particularly heat-sensitive (Knox, 1953).

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A Taxonomic Study of Certain Bacteria Currently Classified as *Vibrio* Species

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SUMMARY

Twenty-five strains currently classified into 14 serological types or species of the genus *Vibrio* obtained from the NCIB and NCTC have been studied in detail for morphological, cultural and biochemical characters in two independent studies. The results indicate that these strains include members of three distinct taxonomic groups or genera, *Vibrio*, *Pseudomonas* and *Comamonas*. The new generic name *Comamonas*, type species *C. percolans*, replaces *Lophomonas* (Galarneault & Leifson) which is invalid.

INTRODUCTION

Thirty-four species are listed in the genus *Vibrio* of *Bergey's Manual* (1957). These species are so heterogeneous in character that it is impossible to define the genus in exclusive terms.

In recent years several new diagnostic tests have been developed for use in bacterial classification, especially of the Gram-negative, polarly flagellate bacteria. By applying a large range of tests to a small number of strains of so-called vibrios, we hoped to clarify the characters of the genus *Vibrio* and appraise the true taxonomic relationships of certain species at present included in the genus.

The work to be described was carried out as independent studies by the two authors. One of us (R.W.A.P.) examined a group of vibrios as part of a comparative study of the polarly flagellate bacteria commonly found in surface waters (see Park, 1961*a*). The other (G.H.G.D.) was asked by Dr R. Cain to determine the taxonomic relationships of a so-called vibrio (vibrio 01, Happold & Key, 1932) commonly used in biochemical studies, e.g. Cain (1961). In order to do this a number of other vibrio strains were obtained for comparative study. Although basically similar, the two studies differ in the tests, techniques and strains used and sometimes in the results obtained, but, as we hope to show, the conclusions which can be drawn from both studies are in good agreement. The simplest way of presenting our information is to divide each of the sections of this paper dealing with Methods and Results into two parts, distinguished by the initials of the author concerned.

METHODS. 1. (R.W.A.P.)

Strains examined

Vibrio comma (Inaba), NCTC 4693.
Vibrio sp. (Gardner & Venkatraman group III), NCTC 4711.
Vibrio sp. (Gardner & Venkatraman group V), NCTC 4715.

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Vibrio sp. (Gardner & Venkatraman group VI), NCTC 4716.

Vibrio sp. (Gardner & Venkatraman group II), NCTC 8042.

V. eltor NCTC 5395.

V. paracholerae (Gardner & Venkatraman group III), NCTC 30.

V. alcaligenes (Lehmann & Neumann, 1931), NCTC 9239.

V. percolans (Mudd & Warren, 1923), NCTC 1937.

V. proteus (Buchner, 1885), NCTC 8563.

V. cuneatus (Gray & Thornton, 1928), NCIB 8194.

V. cyclosites (Gray & Thornton, 1928), NCIB 2581.

V. neocistes (Gray & Thornton, 1928), NCIB 2582.

Vibrio 01 (Happold & Key, 1932), NCIB 8250.

Strain maintenance

Stock cultures were grown 24 hr. on nutrient agar slopes in loosely closed screw-capped bottles and stored at room temperature with the caps screwed down; fresh cultures were prepared monthly.

Incubation at 30° was used throughout this study, except in certain tests as indicated. Storage at room temperature was necessary because *Vibrio comma* does not survive well at low temperatures, e.g. 4°.

Characters examined

1. Cell-form and Gram-reaction of 24 hr. nutrient agar growth.

2. Occurrence and type of motility in hanging-drop preparations of 18 hr. peptone water culture.

3. Disposition and number of flagella using electron microscopy.

4. Metabolism of carbohydrates:

(i) Acid and gas production from the following carbohydrates: DL-arabinose, D-glucose, D-fructose, D-mannose, D-galactose, mannitol, dulcitol, sorbitol, sucrose lactose, maltose, salicin; tested in 1 % (w/v) concentration in peptone water (Mackie & McCartney, 1953) and also in a dilute meat extract medium (Simon, 1956), both media containing bromothymol blue as pH indicator. Arabinose was added as a Seitz-filtered solution to sterile base: gas production from this sugar was not tested for. All other carbohydrate media were steam sterilized. Control tests without carbohydrate were also inoculated. Initial pH of all tests was 7·4, and tests were read after 24 hr. and 7 days' incubation. As results were identical by both methods, only one set has been recorded.

(ii) Utilization of carbohydrates as sole carbon sources. Tested upon following medium (%, w/v): NH$_4$Cl, 0·1; K$_2$HPO$_4$, 0·1; MgSO$_4$, 0·05; agar, 1·5. Same carbohydrates used as in (i) above, at same concentration, all added as Seitz-filtered solutions. Utilization was inferred from the growth of an organism within 7 days in two successive subcultures. Controls without carbohydrate were included.

(iii) Oxidation *versus* fermentation of glucose; test as described by Hugh & Leifson (1953), initial pH 7·4.

(iv) Production of reducing compounds from gluconate (Haynes, 1951). Medium (%, w/v): Oxoid peptone, 0·2; Oxoid yeast extract, 0·1; K$_2$HPO$_4$, 0·1; pH 7·0. Basal medium autoclaved and Seitz-filtered 40 % (w/v) aqueous potassium gluconate

solution added to give final concentration of 4% (w/v). Dispensed aseptically, inoculated and shaken by hand twice daily during 5 days' incubation. Tested with Benedict's qualitative reagent.

(v) Acetylmethylcarbinol production from glucose, using Barritt's (1936) method, but incubated for 5 days.

(vi) Methyl-red test after 5 days' incubation (Mackie & McCartney, 1953).

(vii) Modified Eijkman test (*Topley & Wilson's Principles*, 1955).

(viii) Hydrolysis of starch within 5 days, tested upon nutrient agar containing 0·2% (w/v) soluble starch by flooding with iodine solution (Allen, 1918).

5. Nitrate reduction, tested in 5-day-old peptone water cultures containing 0·1 % (w/v) KNO_3, using the Griess-Ilosvay reagents and zinc powder test for false negatives (ZoBell, 1932).

6. Cholera-red reaction, tested by adding conc. H_2SO_4 (0·5 ml.) to a 5-day old culture in peptone water containing 0·001% (w/v) KNO_3 (Beam, 1959).

7. Indole production, tested in 5-day-old peptone (Oxoid) water cultures using Ehrlich reagent and ether extraction.

8. Utilization of tryptophan, phenylalanine, phenol, benzoic acid, and catechol as sole carbon sources. Compounds were supplied in 0·1 % (w/v) concentration in the inorganic base described in 4 (ii) above. (Note: phenol was used in 0·05% (w/v) as 0·1 % inhibited certain strains; autoxidation of catechol by light, heat or high pH was avoided by adjusting basal medium to pH 6·5 and sterilizing by Seitz-filtration.)

9. Tyrosine breakdown, tested by the ability to dissolve tyrosine (0·5%, w/v) suspended in nutrient agar within 7 days.

10. Phenylalanine and malonate breakdown, tested by the method of Shaw & Clarke (1955).

11. Gelatin hydrolysis, using 0·5% (w/v) gelatin in nutrient agar and flooding 5-day growth with acid mercuric chloride (Frazier, 1926).

12. Serum hydrolysis, observed after 24 hr. and 7 days' growth upon inspissated serum.

13. Casein hydrolysis, using Hastings' (1904) method with 10% (v/v) skimmed milk in nutrient agar; examined after 5 days.

14. Hydrogen sulphide production from peptone water, detected with lead acetate paper; examined after 24 hr. and 7 days.

15. Urea breakdown within 7 days, using Christensen (1946) medium in liquid form. Controls without area were included.

16. Tributyrin hydrolysis, detected by clearing of 0·2% (v/v) tributyrin in nutrient agar after 24 hr. and 7 days.

17. Utilization of organic acids as sole carbon sources, inferred from growth within 7 days in two successive subcultures upon the inorganic medium described in 4 (ii) above containing 0·25% (w/v) of acid. Tested: succinic, malic, acetic, citric, pyruvic, lactic, malonic, and formic acids, all supplied as Seitz-filtered solutions of the sodium salts.

18. Utilization of ethanol and production of excess acid was tested by growth within 7 days in two successive subcultures upon inorganic medium (4 ii above) containing 1 % (w/v) calcium carbonate and 1 % (v/v) ethanol. Excess acid production was shown by clearing of $CaCO_3$ around growth. Controls without ethanol

were incubated separately. (Note: this is not the test of Shimwell, Carr & Rhodes, 1960, as used by G. H. G. D., Methods, 2.)

19. Catalase production, tested with 10 vol. H_2O_2 upon 24 hr. nutrient agar growth.

20. Kovacs (1956) oxidase test.

21. Cytochrome oxidase test of Gaby & Hadley (1957).

22. Growth in peptone water at 0° (within 14 days), 30°, 37°, 42°, 45° (within 7 days). Tested twice.

23. Sodium chloride tolerance, tested by growth within 7 days in peptone water containing 0·5, 1, 3, 6 or 9 % (w/v) NaCl. Tested in triplicate.

24. Growth upon MacConkey agar (Oxoid) and Aronson (pH 9) medium (Mackie & McCartney, 1953) within 7 days.

25. Pyocyanin production. In addition to routine observation of all cultures, 7-day growth upon the glycerol-peptone medium of Gessard (1891) was examined for diffusible pigment in daylight and under a source of ultraviolet radiation fitted with a Wood's glass (u.v.).

26. Fluorescin production. In addition to routine observation, strains were tested for this character in the medium of Georgia & Poe (1931) and upon the inorganic base described in 4 (ii) above containing D-glucose (1 %, w/v) and $CaCO_3$ (1 %, w/v). Cultures were examined after 1, 3, and 7 days in daylight and under u.v.

27. Resistance to antibacterial agents. Tested: penicillin, 2·5 i.u.; Terramycin, 10 μg.; streptomycin, 80 μg.; chloramphenicol, 100 μg., and 2:4-diamino 6:7-di-*iso*propyl pteridine (vibriostatic agent O/129). Inhibition of growth at 24 hr. by these substances was tested upon nutrient agar seeded with organisms using Evans 'Sentest' disks (antibiotics) or, in the case of O/129, a few crystals placed upon the agar surface.

METHODS. 2. (G.H.G.D.)

Strains examined

Vibrio comma, NCTC 4693, NCTC 6585, NCTC 7270, NCTC 8021, NCTC 8367.
V. percolans, NCIB 8193.
V. cuneatus, NCIB 8194.
V. cyclosites, NCIB 2581.
V. neocistes NCIB 2582.
V. 01, NCIB 8250.

Strain maintenance

As R.W.A.P., but using cotton-wool-plugged tubes and subcultured every 2 weeks.

Characters examined

1. Cell-form and Gram-reaction, as R.W.A.P.

2. Motility in nutrient broth at 25°, observed by phase-contrast microscopy after 1–3 days.

3. Flagellation, as R.W.A.P., and various staining methods.

4. Carbohydrate metabolism:

(i) Acid production. Basal medium: Koser salt solution (Mackie & McCartney, 1953, p. 429) plus Oxoid yeast extract 0·3 % (w/v). Initial pH adjusted to 7·0, 2 ml. of 1·5 % (w/v) solution of bromocresol purple added per litre and medium

autoclaved. Carbohydrates were added as 20 % (w/v) autoclaved solutions to give 1 % (w/v) final concentration. The complete media were aseptically dispensed and tested for sterility by incubating for 48 hr. at 30°. Carbohydrates used: arabinose (L +), xylose, glucose, fructose, mannose, galactose, rhamnose, mannitol, sorbitol, dulcitol, inositol, sucrose, maltose, lactose melibiose, raffinose, dextrin, inulin, glycogen, salicin. Tests read daily over 14 days.

(ii) Oxidation *versus* fermentation of glucose. Modified from Hugh & Leifson (1953). Basal medium as in (i) above, plus 1 % (w/v) glucose and 0·3 % (w/v) agar. Initial pH 7·0 and 2 ml. of 1·5 % (w/v) bromocresol purple added per litre. (Note: these modifications were recommended by Dr A. C. Hayward, Commonwealth Mycological Institute, Kew, and Dr A. C. Baird-Parker, Unilever, Bedford, and do appear to clarify the results of this test.)

(iii) Gas from glucose, in addition to observations of (ii) above, routine peptone water fermentation tests containing Durham tubes were observed over 14 days.

(iv) Voges–Proskauer and methyl-red tests as Mackie & McCartney (1953).

(v) Acetylmethylcarbinol (acetoin) production and final pH from 1 % (w/v) glucose in the basal medium of (i) above, initial pH 7·0. Tested after 4 days using pH test papers and Barritt's (1936) method for acetylmethylcarbinol.

(vi) Starch hydrolysis, as R.W.A.P.

(vii) Aesculin hydrolysis within 7 days in the basal medium of (i) above, plus 0·5 % (w/v) aesculin and 0·005 % (w/v) ferric ammonium citrate.

(viii) Dihydroxyacetone production from glycerol, test of Shimwell, Carr & Rhodes (1960).

5. Nitrate reduction: Basal medium 4 (i) above plus 0·1 % (w/v) KNO_3 and 0·1 % (w/v) agar. Procedure as R.W.A.P.

6. Cholera-red test, in Oxoid peptone water (Mackie & McCartney, 1953).

7. Indole production, in 1 % (w/v) Oxoid tryptone water, tested after 3 days by ether extraction and Ehrlich reagent.

8. Tyrosine breakdown, as R.W.A.P.

9. Gelatin hydrolysis, as R.W.A.P., but using 0·2 % (w/v) gelatin.

10. Serum hydrolysis, as R.W.A.P.

11. Casein hydrolysis, as R.W.A.P.

12. Lysine decarboxylase activity. Method of Carlquist (1956). Medium: Basal medium of 4(i) above plus 0·5 % (w/v) lysine hydrochloride. Tested after 5 days.

13. Hide-powder lysis. Method of Evans (1947) in double-layer plates of basal medium 4(i) above with hide-powder (Baird & Tatlock) incorporated in top layer. Examined daily.

14. Hydrogen sulphide production. Basal medium of 4(i) above, plus (i) 0·05 % (w/v) L-cysteine-HCl, or (ii) 0·1 % (w/v) sodium thiosulphate. H_2S detected by lead acetate paper, over 7 days.

15. Ammonia production from arginine. Medium: (%, w/v), Oxoid tryptone, 0·2; NaCl, 0·5; K_2HPO_4, 0·03; L-arginine-HCl, 0·5; glucose, 0·1; pH 7·1. Tested with Nessler reagent after 5 days.

16. Urea breakdown by Christensen's (1946) method, in cotton-wool-plugged tubes, read over 7 days.

17. Egg-yolk reaction, tested as recommended by Willis (1960) after 6 days.

18. Tributyrin hydrolysis, tested by clearing within 7 days of Oxoid glycerol tributyrate (1 %, w/v) agar.

19. Utilization of organic acids as sole carbon sources. Medium: Koser salt solution plus 1·5 % (w/v) agar and 0·008 % bromothymol blue, pH 6·8. Acids added as sodium salts to give 0·25 % (w/v) final concentration. Succinic, malic, acetic, citric, lactic and oxalic acids tested. Tested on slopes, read up to 7 days.

20. Acid production from ethanol, test of Shimwell et al. (1960).

21. Oxidation of calcium lactate to carbonate, test of Shimwell et al. (1960).

22. Catalase production, as R.W.A.P.

23. Oxidase production, as R.W.A.P.

24. Cytochrome oxidase production, as R.W.A.P.

25. Phosphatase production, tested upon nutrient agar containing 0·006 % (w/v) phenolphthalein phosphate, exposed after 5 days to vapour from 0·880 ammonia solution.

26. Growth within 7 days on nutrient agar containing 5 % (w/v) NaCl, 5 or 10 % (w/v) sodium taurocholate; growth and reduction with 0·2 or 1 % (w/v) triphenyl tetrazolium chloride (T.T.C.) (Meitert, Meitert & Horodniceanu, 1960).

27. Growth and characteristics upon MacConkey, Wilson & Blair, Dieudonné and deoxycholate-citrate agars (Mackie & McCartney, 1953), over 7 days.

28. Growth on nutrient agar under strict anaerobiosis, 7 days.

29. Growth in nutrient broth at 4° (14 days), 14°, 25°, 30°, 44° (7 days).

30. Growth from initial pH 4·5, test of Shimwell et al. (1960).

31. Pigment production. Routine observation of all cultures in daylight; fluorescence under u.v. of growth upon the glucose-calcium carbonate medium of R.W.A.P.

32. Resistance to antibacterial agents. Tested using Evans 'Sentest' or Mast disks on surface seeded nutrient agar. Antibiotics: penicillin, 1 and 2·5 i.u.; streptomycin, 20 and 80 μg.; chloramphenicol, 40 and 100 μg.; Aureomycin, 10 and 100 μg.; Terramycin, 10, 25 and 100 μg.; erythromycin, 1 and 10 μg.; tetracycline, 100 μg.; neomycin, 100 μg.; bacitracin, 5·5 units; novobiocin, 5 and 10 μg.; oleandomycin, 5 and 20 μg. Also tested O/129 (see R.W.A.P.) using an aqueous suspension (c. 50 mg. in 20 ml.) and agar well technique. (Note: a saturated aqueous solution of O/129 proved non-inhibitory against *Vibrio comma* in preliminary tests.)

RESULTS

Tables 1 (R.W.A.P.) and 2 (G.H.G.D.) show the results obtained by the two authors and the classification of strains into three groups on the evidence of these results. The overall characters of these three groups are compared in Table 3.

Morphological details are not readily presentable in tables and we therefore include here an account of our observations. The six strains of *Vibrio comma* used by G.H.G.D., and strains 4693 (*V. comma*), 5395 ((*V. eltor*), 8563 (*V. proteus*), 8042, 4711, 4715 and 4716 studied by R.W.A.P. all exhibited pleomorphism. Straight and curved short rods, S-forms, spirals, undulating filaments and spheroplasts (Pitzurra & Szybalski, 1959) were regularly seen. This variety of forms has been reported before in studies upon *V. comma* (Koch, 1886; Henrici, 1925). Plate 1, figs. 1 and 7, illustrates some of these forms. *V. paracholerae* (NCTC 30) did not

Table 1. *Results of R.W.A.P.*

Strain	Vibrio spp.								Pseudo-monas spp.	Comamonas spp.				
	4693	4711	4715	4716	8042	5395	8563	30	8194	2581	2582	1937	9239	8250
Flagellation	1L	1L	1L	1L	1L	1L	1L	1L	M	ML	ML	M	1	1
Acid production and utilization of:														
Arabinose	−	+	−	−	−	−	−	−	+U	−	−	−	−	−
Glucose	+	+	+	+	+	+	+	+	+U	−	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Mannose	+	−	+	+	+	+	+	+	+U	−	−	−	−	−
Galactose	±	+	+	±	+	+	±	+	+U	−	−	−	−	−
Mannitol	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Sucrose	+	+	+	+	+	+	±	−	−	−	−	−	−	−
Lactose	±	±	±	±	+	±	±	±	−	−	−	−	−	−
Maltose	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Salicin	−	−	−	−	+	−	±	−	−	−	−	−	−	−
Hugh & Leifson test	F	F	F	F	F	F	F	F	O	Al.	Al.	Al.	Al.	Al.
Gluconate test (Haynes)	−	+	+	+	−	−	−	+	−	−	−	−	−	−
Acetoin production	−	+	+	+	+	+	−	−	−	−	−	−	−	−
Methyl-red test	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Starch hydrolysis	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	−	+	−	−	−	+	+	−
Cholera-red test	+	+	+	−	+	−	−	+	−	−	−	−	−	−
Indole production	+	+	+	+	+	−	+	+	−	−	−	−	−	−
Utilization of:														
Tryptophan	−	−	−	−	−	−	−	−	+B	−	−	−	−	+
Phenylalanine	−	−	−	−	−	−	−	−	+	−	−	−	−	+
Phenol	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Benzoic acid	−	−	−	−	−	−	−	−	+	−	−	−	−	+
Catechol	−	−	−	−	−	−	−	−	+	−	−	−	−	+
Tyrosine dissolution	+	+	−	−	−	−	−	−	+	−	+	+	+B	+
Phenylalanine (Shaw & Clarke)	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	−	−	−	+	−
Serum hydrolysis	±	+	+	−	±	+	±	±	−	−	−	−	−	−
Casein hydrolysis	+	+	+	+	+	+	+	+	−	−	−	−	−	−
H₂S from peptone	±	±	−	±	±	−	−	±	−	−	−	±	−	−
Urease	−	−	−	−	−	−	−	−	±	−	+	+	−	−
Utilization of:														
Succinic	+	+	+	+	+	+	+	−	+	−	−	−	−	+
Malic	+	+	+	+	+	+	+	−	+	−	−	−	−	+
Acetic	+	−	−	+	−	+	+	−	+	−	−	−	−	+
Citric	−	+	+	+	+	+	+	−	+	−	−	−	−	+
Pyruvic	+	−	+	+	+	+	+	−	+	−	−	−	−	−
Lactic	+	+	+	+	+	+	−	−	+	−	−	−	−	−
Malonic	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Formic	−	−	−	−	−	−	−	−	+	−	−	−	−	+
Utilization and acid from ethanol	−	−	−	−	−	−	−	−	U	−	−	−	−	UA
Catalase	±	±	±	±	±	±	±	±	+	+	±	±	+	+
Kovacs oxidase	+	+	+	+	+	+	−	−	+	−	−	−	+	+
Cytochrome oxidase	±	±	±	−	−	−	−	−	±	±	±	−	−	−
Growth at 0°	−	−	−	−	−	−	+	−	+	−	−	−	−	−
Growth at 37°	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Growth at 42°	+	+	+	+	−	+	+	+	−	+	+	−	−	−
Growth in 3% NaCl	+	+	+	+	+	+	+	+	+	−	−	−	+	−
Growth in 6% NaCl	−	−	+	−	−	−	+	−	−	−	−	−	−	−
MacConkey	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Aronson	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Fluorescin	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Fluorescence u.v.	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Susceptible to:														
Penicillin 2·5	+	−	−	±	−	±	±		+	+	+	+	−	−
Streptomycin 80	+	+	+	+	+	+	+	−	+	+	+	+	−	+
O/129	+	+	+	+	+	+	+	−	−	−	−	−	−	−

Key, see p. 119.

Table 2. *Results of G.H.G.D.*

Strain	Vibrio spp.						Pseudomonas spp.	Comamonas spp.			
	4693	6585	7270	7254	8021	8367	8194	2581	2582	8193	8250
Flagellation	1L	1L	1L	1L	1L	1L	M	M	M	M	—
Acid from:											
Arabinose	—	—	—	—	—	—	7	10	—	—	—
Xylose	—	—	—	—	—	—	3	—	—	—	—
Glucose	3	3	3	3	3	3	3	12	—	—	—
Fructose	3	3	3	3	3	3	10	10	—	—	—
Mannose	3	3	3	3	3	3	3	14	—	—	—
Galactose	—	3	3	3	3	3	3	14	—	—	—
Rhamnose	—	—	—	—	—	—	—	7	—	—	—
Mannitol	7	3	3	3	3	3	7	10	—	—	—
Inositol	—	—	—	—	—	—	7	—	—	—	—
Sucrose	3	3	3	3	3	3	5	7	—	—	—
Maltose	3	3	3	3	3	3	—	14	—	—	—
Lactose	—	7	12	7	12	—	—	12	—	—	—
Melibiose	—	—	—	—	—	—	3	12	—	—	—
Dextrin	3	3	3	3	3	3	—	10	—	—	—
Glycogen	3	3	3	3	3	3	—	—	—	—	—
Salicin	—	—	—	—	—	—	—	12	—	—	—
Hugh & Leifson test	F	F	F	F	F	F	O	O±	—	—	—
Methyl-red test	+	+	+	+	+	+	—	—	—	—	—
Acetoin	—	—	±	—	—	—	—	—	—	—	—
pH 1 % glucose	5·1	5·1	5·1	6·1	5·1	4·5	6·1	6·7	6·7	7·1	7·1
Starch hydrolysis	+	+	+	+	+	+	—	±	—	—	—
Aesculin hydrolysis	—	—	—	—	—	—	—	+	—	—	—
Nitrate reduction	+	+	+	+	+	+	+	+	+	—	—
Cholera-red test	+	+	+	+	+	+	—	—	—	—	—
Indole	+	+	+	+	+	+	—	—	—	—	—
Tyrosine dissolution	±	—	±	—	—	—	+	—	+	+	±
Gelatin hydrolysis	+	+	+	+	+	+	+	—	—	—	—
Serum hydrolysis	—	+	+	+	+	+	+	—	—	—	—
Casein hydrolysis	+	+	+	+	+	+	—	±	—	—	—
Lysine decarboxylase	+	+	+	+	+	+	—	—	—	—	—
Hide-powder lysis	+	+	+	+	+	+	—	—	—	—	—
H$_2$S cysteine	+	+	+	+	+	—	±	±	±	±	±
H$_2$S thiosulphate	+	+	+	+	+	—	—	±	±	±	—
NH$_3$ from arginine	—	—	—	—	—	—	+	—	—	—	—
Urease	—	—	—	—	—	—	+	—	+	+	—
Egg yolk reaction	Li	Li	Li	Li	Li	Li	Li	—	—	—	Li±
Tributyrin	NG	NG	NG	NG	NG	NG	+	+	+	+	+
Utilization of											
Malic	+	+	+	+	+	±	+	—	+	+	+
Acetic	+	+	+	+	+	±	+	—	+	+	+
Lactic	+	+	+	+	+	+	+	—	+	+	+
Oxalic	—	—	—	—	—	—	—	—	—	+	—
Acid from ethanol	—	—	—	—	—	—	—	—	—	—	±
Lactate oxidation	—	—	—	—	—	—	—	—	—	—	+
Catalase	±	±	±	±	±	±	+	+	+	+	+
Kovacs oxidase	+	+	+	+	+	+	+	—	+	+	—
Cytochrome oxidase	+	+	+	+	+	+	—	—	±	—	—
Phosphatase	—	—	—	—	—	—	+	—	—	—	±
5 % NaCl tolerance	+	±	—	+	—	+	±	+	—	—	±
5 % Bile tolerance	±	+	+	+	+	+	+	—	+	±	+
10 % Bile tolerance	±	—	+	+	+	+	+	—	—	—	+

Key, see p. 119.

Table 2 (*cont.*)

Strain	Vibrio spp.						Pseu-domo-nas spp.	Comamonas spp.			
	4693	6585	7270	7254	8021	8367	8194	2581	2582	8193	8250
0·2 % T.T.C. tolerance	−	−	+	−	+	−	+	−	+	+	+
1 % T.T.C. tolerance	−	−	−	−	−	−	+	−	−	−	−
MacConkey	+	+	+	+	+	+	+	−	+	+	+
Wilson and Blair	−	+	+	+	+	+	+	−	+B	+	+B
Dieudonné	+	+	+	+	+	+	±	−	−	−	−
Deoxycholate-citrate	−	+	+	+	+	+	+	−	−	−	−
Anaerobic growth	±	±	±	±	±	±	−	−	−	−	−
Growth at 4°	−	−	−	−	−	−	+	±	−	−	±
Growth at 14°	+	+	+	+	+	+	+	±	+	±	+
Growth at pH 4·5	−	−	−	−	−	−	+	−	+	+	+
Pigment daylight	−	−	−	−	−	−	YG	Y	−	−	−
Fluorescence, u.v.	−	−	−	−	−	−	+	−	−	−	−
Susceptible to											
Chloramphenicol 100	+	+	+	+	+	+	±	+	+	±	+
Chloramphenicol 40	+	+	+	+	+	+	±	−	+	−	+
Terramycin 10	+	+	+	+	+	+	+	+	+	±	+
Tetracycline 100	+	+	+	±	+	+	+	+	+	+	±
Aureomycin 100	+	+	+	+	+	+	±	+	+	+	±
Aureomycin 10	+	+	+	+	+	+	−	+	+	+	−
Streptomycin 80	+	+	+	+	+	+	+	+	+	−	+
Streptomycin 20	+	+	+	+	+	+	+	+	+	−	+
Neomycin 100	+	+	+	+	+	+	+	+	+	−	−
Erythromycin 10	+	+	+	+	+	+	−	+	−	−	−
Erythromycin 1	+	+	+	−	+	±	−	±	−	−	−
Novobiocin 10	+	+	+	+	+	+	−	+	−	−	−
Novobiocin 5	+	+	+	+	+	+	−	−	−	−	−
Oleandomycin 20	+	+	+	−	+	±	−	+	−	−	−
O/129	±	±	+	−	±	±	−	−	−	−	−

Key, see p. 119.

show the same degree of pleomorphism, only straight and curved rods and sphero-plasts were seen (Pl. 1, fig. 2). All these strains exhibited single polar flagella upon the short cell forms and often had a flagellum at both poles of the longer forms, some of which appeared to be dividing (Pl. 1, fig. 1). Spheroplasts commonly retained flagella but motility of spheroplasts was never observed in living material (cf. Jeynes, 1961).

Strain 8194 (*Vibrio cuneatus*) consisted of robust, round-ended rods which were usually curved (Pl. 1, fig. 4). One or more polar flagella were seen (1–10, R.W.A.P.). No pleomorphism other than variation in cell length was observed and this may correlate with observed colonial variations between smooth soft and rough hard forms. The tapering cell forms described by Gray & Thornton (1928) were not seen.

Strains 2581 (*Vibrio cyclosites*) and 2582 (*V. neocistes*) were observed by R.W.A.P. as fairly robust, straight rods with round ends, usually bearing 2–6 flagella at one pole and one at the other (Pl. 1, fig. 3). Leifson (1960) has illustrated a very similar form (Leifson, fig. 16d) showing division of the organism. Our figure does not show division in progress. G.H.G.D. observed lophotrichous but not bipolar, flagellation in strain 2518. Loss of motility appeared to occur readily in this strain and

coccoid and swollen forms were sometimes seen. Strain 2582 was similar in flagellation to 2581, and regularly motile. Filamentous forms that divided by constriction (Bisset, 1952), and appeared as chains of fusiform cells were observed in strain 2582.

Strains 8193 (G.H.G.D.) and 1937 (R.W.A.P.) (*Vibrio percolans*) were observed by both authors to consist of robust, straight rods with round ends and 1–5 flagella at one pole (Pl. 1, fig. 5). Filamentous forms showing constrictions were common, cf. Leifson (1960, fig. 16c), and strain 2582 above. Similar tendencies to filamentous

Table 3. *Comparison of certain results from R.W.A.P. and G.H.G.D. for the groups indicated in Tables 1 and 2*

Group Author and number of strains	Vibrio R.W.A.P. (8)	Vibrio G.H.G.D. (6)	P. fluorescens (8194) R.W.A.P. (1)	P. fluorescens (8194) G.H.G.D. (1)	Comamonas R.W.A.P. (5)	Comamonas G.H.G.D. (4)
Flagellation	1L	1L	M	M	1, M or ML	3/4 M
Acid from						
Arabinose	−	−	+U	+	−	1/4±
Xylose	NT	−	NT	+	NT	−
Glucose	+	+	+U	+	−	1/4±
Fructose	+	+	−	±	−	1/4±
Mannose	7/8	+	+U	+	−	1/4±
Galactose	+ or ±	5/6	+U	+	−	1/4±
Rhamnose	NT	−	NT	−	NT	1/4±
Mannitol	+	+	−	±	−	1/4±
Inositol	NT	−	NT	±	NT	−
Sorbitol	1/8	−	−	−	−	−
Sucrose	7/8	+	−	+	−	1/4±
Lactose	±	4/6±	−	−	−	1/4±
Maltose	+	+	−	−	−	1/4±
Melibiose	NT	−	NT	+	NT	1/4±
Dextrin	NT	+	NT	−	NT	1/4±
Glycogen	NT	+	NT	−	NT	−
Salicin	2/8	−	−	−	−	1/4±
Hugh & Leifson test	F	F	O	O	Al	1/4±O
Methyl-red test	2/8	+	−	−	−	−
Acetoin	5/8	1/6	−	−	−	−
pH 1 % glucose	NT	below 6	NT	7·1	NT	c. 7
Starch hydrolysis	+	+	−	−	−	1/4±
Aesculin hydrolysis	NT	−	NT	−	NT	1/4±
Nitrate reduction	7/8	+	−	+	2/5	2/4
Cholera-red test	5/8	+	−	−	−	−
Indole	7/8	+	−	−	−	−
Tyrosine dissoluton	2/8	2/6±	+	+	4/5	3/4
Gelatin hydrolysis	+	+	+	+	1/5	−
Serum hydrolysis	7/8±or+	5/6	+	+	1/5	−
Casein hydrolysis	+	+	−	−	−	1/4±
Lysine decarboxylase	NT	+	NT	−	NT	−
Hide-powder lysis	NT	+	NT	−	NT	−
H₂S cysteine	NT	+	NT	±	NT	±
H₂S thiosulphate	NT	+	NT	−	NT	3/4±
H₂S peptone	5/8±	NT	−	NT	1/5±	NT
NH₃ from arginine	NT	−	NT	+	NT	−
Urease	−	−	±	+	2/5	2/4
Tributyrin 0·2 %	+	NT	+	NT	+	NT
Tributyrin 1 %	NT	NG	NT	+	NT	+
Egg-yolk reaction	NT	Li	NT	Li	NT	1/4 Li±

Key, see p. 119.

Table 3 (*cont.*)

Group Author and number of strains	*Vibrio* R.W.A.P. (8)	*Vibrio* G.H.G.D. (6)	*P. fluorescens* (8194) R.W.A.P. (1)	*P. fluorescens* (8194) G.H.G.D. (1)	*Comamonas* R.W.A.P. (5)	*Comamonas* G.H.G.D. (4)
Utilization of:						
Tryptophan	−	NT	+	NT	1/5	NT
Phenylalanine	−	NT	+	NT	−	NT
Phenol	−	NT	−	NT	1/5	NT
Benzoic acid	−	NT	+	NT	1/5	NT
Catechol	−	NT	−	NT	1/5	NT
Phenylalanine (Shaw & Clarke)	−	NT	−	NT	1/5	NT
Malonate (Shaw & Clarke)	+	NT	+	NT	+	NT
Succinic	7/8	+	+	+	1/5	+
Malic	7/8	+	+	+	1/5	3/4
Acetic	4/8	+	+	+	1/5	3/4
Citric	6/8	+	+	+	1/5	+
Pyruvic	6/8	NT	+	NT	−	NT
Lactic	6/8	+	−	−	−	3/4
Oxalic	NT	−	NT	−	NT	1/4
Malonic	−	NT	+	NT	−	NT
Formic	−	NT	+	NT	1/5	NT
Utilization of ethanol	−	NT	+	NT	1/5	NT
Acid from ethanol	−	−	−	−	1/5	1/4
Lactate oxidation	NT	−	NT	−	NT	1/4
Catalase	±	±	+	+	+ or ±	+
Kovacs oxidase	6/8	+	+	+	2/5	2/4
Cytochrome oxidase	3/8±	+	±	−	2/5±	1/4±
Phosphatase	NT	−	NT	+	NT	1/4±
NaCl tolerance:						
3%	+	NT	+	NT	1/5	NT
5%	NT	4/6	NT	±	NT	2/4
10% bile tolerance	NT	5/6	NT	+	NT	1/4
1% T.T.C. tolerance	NT	−	NT	+	NT	−
Growth at:						
0°	1/8	NT	+	NT	−	NT
4°	NT	−	NT	+	NT	2/4±
42°	6/8	NT	−	NT	2/5	NT
MacConkey	+	+	+	+	4/5	3/4
Aronson	+	NT	−	NT	−	NT
Dieudonné	NT	+	NT	±	NT	−
Wilson & Blair	NT	5/6	NT	+	NT	3/4
Deoxycholate citrate	NT	5/6	NT	+	NT	−
Anaerobic growth	NT	±	NT	−	NT	−
Growth at pH 4·5	NT	−	NT	+	NT	3/4
Fluorescence u.v.	−	−	+	+	−	−
Pigment, daylight	−	−	+	+	−	1/4
Susceptible to O/129	7/8	5/6	−	−	−	−

Key, see p. 119.

growth were observed by R.W.A.P. in certain freshly isolated water bacteria classified as *Comamonas* sp. (Park, 1961 *a*). Variation from polar to peritrichous flagellation as described by Leifson & Hugh (1953) for this organism was not observed, although in a preparation stained by Leifson's (1960) method, many cells

exhibited flagellation similar to that described as 'degenerate peritrichous' by Conn & Wolfe (1938), or 'subpolar' by Leifson (1960). When originally isolated by Mudd & Warren (1923) this organism was found to display a range of morphological forms including what may have been spheroplasts.

Strain 8250 (*Vibrio* 01) had a regular cell form of single and paired, short, thick, straight rods. They appeared to be square-ended in cell-wall stained preparations. R.W.A.P. observed a few motile organisms in peptone water cultures and found occasional organisms bearing one or two flagella at one pole by electron microscopy. Motility was presumably a character of this strain when originally isolated (Happold & Key, 1932) but it appears to be easily lost. No motility was detected by G.H.G.D. and no flagella demonstrated.

Strain 9239 (R.W.A.P.) (*Vibrio alcaligenes*) consisted of round-ended straight rods having one or two flagella at one pole. No other forms were seen (Pl. 1, fig. 6).

Table 4. *Comparison of* Vibrio, Aeromonas, Pseudomonas *and* Comamonas
reactions in possible diagnostic tests

	Vibrio	Aeromonas	Pseudomonas	Comamonas
Spheroplasts regularly formed	+	−	−	−
Pleomorphic	+	−	−	−
Single polar flagellum	+	V	V	V
Fluoresce under u.v.	−	−	+	−
Growth at pH 9	+	V	V	V
Susceptible to O/129	+(V)	− ?	−	−
Hugh & Leifson test	Ferment	Ferment	Oxidize	Alkaline or Nil
Gas from glucose	−	+(V)	−	−
Cholera-red test	V	−	−	−

Key: + = positive; − = negative; V = some strains positive; +(V) = most strains positive; ? = see Rhodes (1959) and text.

Note. This table is based on our own results, see Park (1961a), and those of previous workers.

Detailed comparisons of our other results can readily be made from the tables we have given. One or two features of these results will serve as examples.

Differences in the results of Kovacs oxidase test and the cytochrome oxidase test in the two studies probably reflect a lack of definition in the execution and reading of these tests. One of us (G.H.G.D.) found some variation in results of the cytochrome oxidase test when the strains were tested in various ways, e.g. young and old, broth and agar cultures. Agar cultures tested by the filter-paper technique gave the clearest results. On the other hand, differences in the results for tributyrin attack are explained by the use of different tributyrin concentrations by the two authors (see Methods). It is interesting to note that all six strains of *Vibrio comma* tested by G.H.G.D. failed to grow in 7 days on 1% (w/v) tributyrate agar (cf. Collins & Hammer, 1934).

Good general agreement in carbohydrate metabolism tests is noticeable. The detection of weak oxidation of glucose by strain 2581 (G.H.G.D.) and late attack of various sugars by the same strain may be explained by the absence of peptone from the basal medium used (cf. Liu, 1952). Ammonia production from peptone or arginine was not detected for this strain (G.H.G.D.), but an alkaline reaction was recorded in the standard Hugh & Leifson test (R.W.A.P.). The strain grew in peptone

water which indicates that peptone was attacked, and breakdown products could have masked weak acid production in the tests of R.W.A.P. This does not, however, explain the fact that R.W.A.P. obtained exactly similar results in Simon medium (see Methods). It is possible that the yeast extract used in the medium of G.H.G.D. may have given rise to false late positive results, and it is also possible that differences in the initial pH value and pH indicators employed may have contributed to the different results obtained. Strains 2581 and 2582 were originally described as producing acid from glucose (Gray & Thornton, 1928). R.W.A.P. found that strain 9239 produced an acid reaction in peptone water and also in all the carbohydrate-peptone water media except those containing sucrose or maltose, which became very alkaline. These findings emphasize the need for controls when peptone water is used as a base for carbohydrate breakdown tests (cf. also Orcutt & Nutting, 1942).

The attack on hide powder by *Vibrio comma* confirmed to some extent the report by Narayanan & Menon (1952) of collagenase activity in this organism. This character is fairly common in other Gram-negative polarly flagellate bacteria.

In our results the egg-yolk reaction of *Vibrio comma* was interpreted as due to lipolysis. The 'pearly layer' effect of Willis (1960) was evident, and zones of what appeared to be a crystalline precipitate which reacted with copper sulphate were produced. Felsenfeld (1944) reported lecithinase activity in *V. comma*.

The test for lysine-decarboxylase activity yielded clear results and may be of taxonomic value (Szturm-Rubinsten, Piéchaud & Piéchaud, 1960). Thibauit & Le Minor (1957) reported the use of this test for the differentiation of enterobacteria.

Preliminary analyses of the cell-wall amino-acid components of strains 4693, 8250 and 8194 by the technique of Davis & Freer (1960) indicated gross similarity between these strains and various other Gram-negative bacteria (e.g. *Neisseria* sp., coliforms, etc.). Qualitative differentiation was not possible and all strains so far examined yielded typical Gram-negative, i.e. complex, amino acid patterns.

DISCUSSION

In this study we have applied a large number and variety of simple tests to a small number of bacterial strains representing all but two (*Vibrio fetus*, see Park, 1961*b*, and *V. metschnikovii*) of the so-called vibrios available in the National Collection of Industrial Bacteria and National Collection of Type Cultures. Although the results obtained by the two authors differ in details, the classification of strains derived from these two independent studies is the same. Tables 1 and 2 show how the strains were analysed into groups; the first group in each table represents what we consider to be true members of the genus *Vibrio*, the second (strain 8194) exhibits characters typical of *Pseudomonas fluorescens*, while the third group is comprised of strains that we regard as representatives of the genus named *Lophomonas* by Galarneault & Leifson (1956). Before proceeding to discuss our results it is important to explain that a change of nomenclature is necessary regarding the genus *Lophomonas* (Galarneault & Leifson). In 1860 Stein gave the name *Lophomonas* to a genus of protozoa found in the cockroach colon; see Wenyon (1926) and Kudo (1954) concerning current usage of the name. Under Rule 24 (see also Principle 3 and Opinion 14) of the *International Code of Nomenclature of Bacteria and Viruses* (1958) such a name must not be used in bacteriology because it is a 'later homonym of the name

of a protozoa taxon'. We propose the name *Comamonas* (Latinized Greek: *Coma*—a lock of hair, and *monas*—a unit) to replace *Lophomonas* in bacteriology. The type species of *Lophomonas*, *L. alcaligenes* proposed by Galarneault & Leifson was presumably a new combination of the name *Vibrio alcaligenes* (Lehmann & Neumann, 1931) but this is not only uncertain but also invalid in view of certain differences in definition between *Lophomonas* and *Vibrio alcaligenes* (see Park, 1961 a). We therefore designate the type species of *Comamonas* as *C. percolans* (Mudd & Warren, 1923) *nov. comb.* Cultures of the type strain of this species are maintained as ATCC 8461, NCTC 1937 and NCIB 8193. It is interesting to note here that *Bergey's Manual* (1957) suggests that *Vibrio percolans* and *V. alcaligenes* may be synonymous but no definite information on this point is given.

Strain 8194 is clearly distinct from *Vibrio comma* and as Hugh & Leifson (1953) have pointed out, there seems no justification for retaining this strain in the genus *Vibrio*. We see no reason why this organism should not be reclassified as a strain of *Pseudomonas fluorescens*. See Rhodes (1959) & Park (1961 a) for comparable studies upon other strains of *P. fluorescens*.

The organisms of our third group gave either negative or weak positive results in most of the tests tried, and because they resembled each other in this respect we consider them sufficiently alike to be included in the genus *Comamonas*. Upon the available evidence we cannot decide whether only one species. i.e. *Comamonas percolans*, is represented but this seems probable. Certain variations between strains and even within single strains were noticeable in this group and these suggest a gradation toward the typical *Pseudomonas*. Strain 8250 displayed an ability to utilize various aromatic compounds, while strain 2581 showed some evidence of carbohydrate attack. Similar but more marked gradations towards *Pseudomonas* have been noted independently in other strains of *Comamonas* by both authors, e.g. Park (1961 a). We thus conclude that we can detect in *Comamonas* a continuation of the trend towards inactivity, as judged by the biochemical tests currently used, that was observed by Rhodes (1959) in the phytopathogenic strains of *Pseudomonas*. None of the *Comamonas* strains examined in this work had any history of pathogenicity, but the production of yellow pigment by 2581 (notably on milk agar) is of interest in this connexion. In spite of these considerations we feel that it is taxonomically useful to place in a separate genus, *Comamonas*, those polarly flagellate bacteria that neither produce a compound which fluoresces under u.v. nor show any clearly defined attack on carbohydrates.

The three so-called vibrios of Gray & Thornton (1928), strains 8194, 2581, and 2582, gave different results in some cases from those originally reported for these strains. Of chief interest is probably the failure of the phenol-utilizing *Vibrio cyclosites* (2581) to utilize phenol in our test. It is to be expected, however, that after 30 years of laboratory maintenance these strains might show some changes in their reactions. We consider that although such changes may have occurred, the strains currently labelled as *V. cyclosites*, *V. neocistes* and *V. cuneatus* represent Gray & Thornton's original isolates. Gray & Thornton's classification was acknowledged to be 'one of convenience only and its temporary nature recognized'; over reliance upon their results from diagnostic tests is probably best avoided.

The utilization of oxalate by strain 8193 recorded above indicates a possible relationship with those oxalate decomposers of the *Comamonas* type reported

by Bhat & Barker (1948) and Jayasuriya (1955). In view of our recommendation to rename this organism *Comamonas percolans* as type species of the genus, it is important also to note here that Leifson & Hugh (1953) and R.W.A.P., using cultures ATCC 8461 and NCTC 1937, respectively, found that it reduced nitrate to nitrite, whereas Mudd & Warren (1923) and G.H.G.D., using the original isolate and NCIB 8193, respectively, found no nitrate reduction. The isolation of a peritrichous variant by Leifson & Hugh (1953) has already been mentioned.

Strain 8250 exemplifies some of the taxonomic difficulties which organisms of this type may create. If polar flagellation had not been detected the strain could have been classified as an *Alcaligenes* or *Protaminobacter* species or as *Achromobacter parvulus*, to quote only a few of the possibilities available in *Bergey's Manual* (1957). Possible relationship to the genus *Acetomonas* (Shimwell *et al.* 1960) was also suspected, but Dr J. G. Carr kindly examined the organism and confirmed that classification in *Acetomonas* or *Acetobacter* was unjustified. (Note: Dr Carr also confirmed the lack of motility in the strain supplied to him by G.H.G.D., and did not detect any acid production from ethanol although the strain did produce irisation on ethanol-chalk agar and grew with ethanol as sole carbon source.)

The main object of this study was to define more accurately the genus *Vibrio*. Our results show that the strains we studied include a clear-cut and compact group, all basically the same as *V. comma* in morphological, cultural and biochemical characters. The overall similarity of the members of this group leads us to conclude that they are members of a single species; the conclusion drawn by Sneath & Cowan (1958) that the genus *Vibrio* might reasonably be downgraded to species rank is of interest in this context.

In Table 4 we have listed certain characters which may have, or are commonly considered to have, diagnostic value in differentiating *Vibrio* from other polar flagellate bacteria. The closest similarity occurs between *Vibrio* and *Aeromonas* (see Park, 1961*a*) and the existence of anaerogenic strains of *Aeromonas* (Crawford, 1954; Pivnick & Sabina, 1957; Park, 1961*a*) further complicates their differentiation. Two characters, pleomorphism and sensitivity to O/129, usually found in *Vibrio* but not in *Aeromonas* strains appear to be of possible diagnostic value and require further assessment.

The distinctive morphology observed in all but one of our *Vibrio* strains appears to be of diagnostic value when present. Its absence, however, does not allow one to exclude an organism from *Vibrio* as it is well known that strains of *V. comma* may exist as almost straight rods (see Pollitzer, 1955). Some bacteria that are biochemically very different from *V. comma* also show this distinctive morphology (Smith & Taylor, 1919; Spencer, 1955; Bryant & Small, 1956; Deas, 1960; Park, 1961*b*). It is difficult to decide whether these organisms should be regarded as close relatives of *V. comma* on morphological grounds, or as not related to it because of their distinct biochemical characters. The presence merely of somatic curvature is of no diagnostic value. It is known to occur in strains of many genera, e.g. *Pseudomonas*, *Aeromonas*, *Comamonas*, *Selenomonas*, etc.

Susceptibility to the vibriostatic agent O/129 first used in systematic work by Shewan, Hodgkiss & Liston (1954) seems, like the distinctive morphology, to be of positive value when present, but of no value when absent. The report by Rhodes (1959) that two NCTC strains of *Aeromonas* were sensitive to a soluble phosphate

derivative of O/129 complicates the issue. It may be that results obtained with the phosphate derivative are not strictly comparable with ours. Whatever the explanation, the fact remains that the distinction between these two genera is not clear. (Note: the authors recognize the value of simple diagnostic procedures in routine identification of bacteria, but suggest that the above comments show how important it is to base such procedures upon sound taxonomic knowledge of the organisms concerned. It is certain that reliance upon single characters, however well authenticated, can lead to errors being made. The literature of *Vibrio* is largely concerned with diagnosis rather than with taxonomy.)

Finally, we should like to draw attention to an important aspect of the work carried out in Asia upon the identification and classification of *Vibrio*. The term 'cholera and cholera-like vibrios' frequently occurs in this literature and there is good reason to doubt the true *Vibrio* nature of some of the bacteria studied. The definition of *Vibrio* commonly applied (Taylor, Pandit & Reed, 1937) and the isolation techniques used, would not guarantee exclusion of all organisms which we now recognize as *Comamonas*, *Pseudomonas* or *Aeromonas* strains. The study of Pandit & Maitra (1938) is a good example. The 'water-vibrios' of Heiberg's (1935) Group VI (non-fermenting strains), the 'non-fermenters' of Gardner & Venkatraman (1935), and Linton's chemical group III (see Linton, 1940) are also interesting in this context. It is difficult in most cases to tell whether the organisms which showed no reaction with the cholera somatic *O* antigen (e.g. Taylor & Ahuja, 1938) were in fact non-vibrios or just non-cholera vibrios. It is similarly difficult to analyse the results of Jackson (1948) who isolated 49 strains of 'vibrio' from 32 of 43 English surface-water samples (cf. Park, 1961*a*). The serological heterogeneity of most of these strains does not necessarily exclude them from *Vibrio*, but neither do the isolation methods nor biochemical results reported ensure that all the strains examined were vibrios. In the circumstances it is easy to see why such workers as Gray & Thornton (1928) allotted dubious organisms to *Vibrio*.

Only by making closer and more detailed taxonomic studies of organisms that workers at present consider as *Vibrio* spp , and by unifying the diverse interests in vibrio-like bacteria from various habitats, e.g., marine, soil, animal, human, can we hope to rectify the situation that exists and has been responsible for the inclusion in the genus *Vibrio* of *Bergey's Manual* (1957) of such a heterogeneous collection of bacteria.

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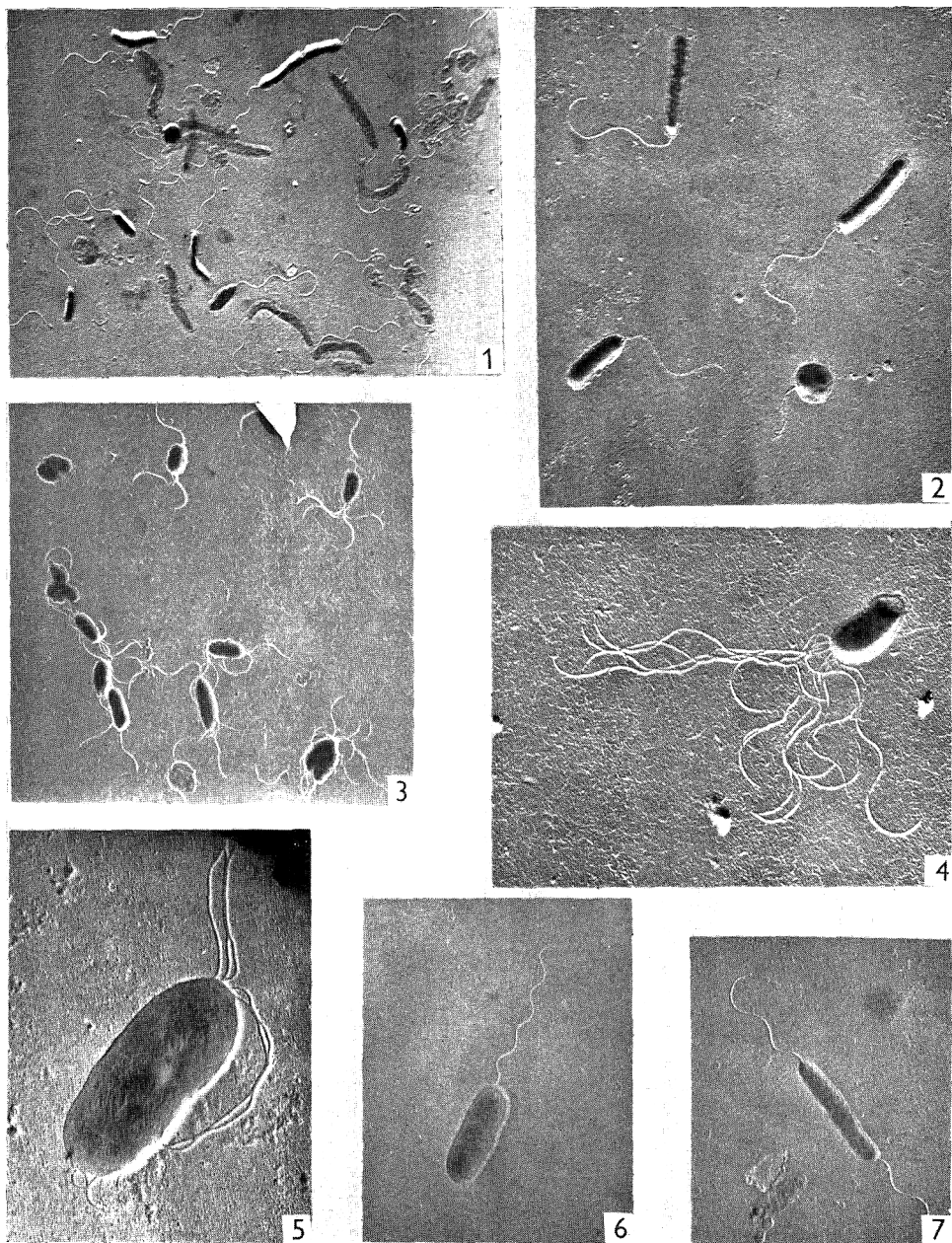
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EXPLANATION OF PLATE

Electronmicrographs; shadowed gold-palladium (60:40) mixture. From 24 hr. growth on nutrient agar.

Figs. 1, 7. Strain 8563 (*Vibrio proteus*) showing straight and curved rods, spheroplasts and long dividing forms, single and bipolar monotrichous flagellation. Fig. 1, × c. 3000; Fig. 7, × c. 4500.

Fig. 2. Strain 30 (*Vibrio paracholerae*) showing rod forms and a spheroplast. × c. 4000.

Fig. 3. Strain 2582 (*Vibrio neocistes, Comamonas* sp.) showing bipolar flagellation as exhibited by this strain and strain 2581. × c. 4500.

Fig. 4. Strain 8194 (*Vibrio cuneatus, Pseudomonas fluorescens*). × c. 6000.

Fig. 5. Strain 1937 (*Vibrio percolans, Comamonas* sp.). × c. 15,000.

Fig. 6. Strain 9239 (*Vibrio alcaligenes, Comamonas* sp.). × c. 5000.

Key to Tables 1–3

Flagellation:

1 = 1 or rarely 2, polar	L = at one or both poles
M = 1 or several, polar	+ = positive
	± = weak, slow or late reaction or growth

Fractional figures = number of strains positive/number tested (in some cases ±/tested).

—	= no reaction, growth, etc.	U = utilized
A	= excess acid	Numbers = day reaction detected
F	= fermentation	O = oxidation
Al.	= alkaline reaction in open tube	Li = lipolysis
NG	= no growth	NT = not tested
YG	= yellow-green	B = black-brown
Y	= yellow	

Notes.

(a) All strains examined were Gram-negative.

(b) In R.W.A.P. results, all strains cleared tributyrin, attacked malonate (Shaw & Clarke), grew at 30° and in 0·5 % and 1 % NaCl, and were susceptible to Terramycin 10 units and Chloramphenicol 100 units. None gave positive Eijkman reaction, utilized or produced acid from dulcitol, grew at 45° or in 9 % NaCl, produced pyocyanin, or gas.

(c) In G.H.G.D. results, all strains grew at 25° and 30°; all utilized succinate and citrate. None produced acid from dulcitol, sorbitol, raffinose or inulin. None produced gas from glucose, gave V.P. + reaction, grew at 44°, produced dihydroxy-acetone from glycerol, were susceptible to bacitracin 5·5 units, oleandomycin 5 µg., penicillin 2·5 and 1 units. All were susceptible to Terramycin 100 and 25 units.

(d) In Table 1, under 'Acid production (and utilization of) :' +, ± and − refer to acid production only.

A Study of Certain Heterotrophic Polarly Flagellate Water Bacteria: Aeromonas, Pseudomonas and Comamonas

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SUMMARY

Sixty-one polarly flagellate bacteria, initially identified as such by the type of motility they displayed, were isolated from surface waters and classified on the basis of cultural and biochemical tests into three genera: *Aeromonas, Pseudomonas, Comamonas*. Sixteen similarly flagellated cultures from other sources were also found to be members of these genera. Some bacteria with a single polar flagellum and giving reactions of the *Alcaligenes* type were isolated. It is suggested that the definition of the genus *Comamonas* (Davis & Park) be extended to include such forms. The possibility of confusing *Aeromonas* with *Vibrio* and with some enterobacteria is discussed.

INTRODUCTION

Although much research into the bacteriology of sea water has been carried out recently there has been little corresponding work on the bacteriology of surface waters, with the exception of studies concerned primarily with public health. Reports on water bacteria made by Ward (1897), Boyce & Hill (1900), Taylor (1942), Thomas & Thomas (1955) and Corberi & Solaini (1960) gave no mention of flagellation or motility in the strains studied, while those of Fuller & Johnson (1899), Jordan (1903) and Cornwall (1914) recorded motility but not flagellation. In fact, so far as the author is aware, only one study on the range of bacteria present in surface waters has included an examination of flagellar pattern (Gray, 1951) but neither the method of determination nor the detailed results obtained were recorded. Therefore, while there is considerable information available about the types of bacteria present in surface waters, it is of limited taxonomic value because the flagellar pattern of strains, a characteristic of importance in current taxonomy, has not been recorded. Shewan, Floodgate & Hayes (1958) noted a similar lack of information in the literature about sea-water bacteria.

Our knowledge of the polarly flagellate Gram-negative bacteria is being increased by studies concerned with the identification and internal classification of one, or sometimes of two genera (Haynes, 1951; Miles & Miles, 1951; Galarneault & Leifson, 1956; Wetmore & Gochenour, 1956; Rhodes, 1959; Eddy, 1960), and by the development of simple methods for differentiating polarly flagellate from peritrichously flagellate and non-motile forms, and for separating polarly flagellate forms into genera (Shewan, Hodgkiss & Liston, 1954; Ewing & Johnson, 1960). However, if a satisfactory classification of the Gram-negative rods is to be achieved, more

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information about the range of types of polarly flagellate bacteria that exist, and about the relationships of these types with one another and with peritrichously flagellate and non-motile bacteria, will have to be obtained. It was thought that a study of polarly flagellate bacteria isolated from surface waters would supply additional information on the flora of this habitat and would enable an assessment to be made of the taxonomic value of a range of biochemical tests when applied to this group.

METHODS

Isolation and recognition of polarly flagellate bacteria

Water samples were taken from ditches, streams and rivers in 100 ml. sterile screw-cap bottles. The bottles were plunged into the water and pushed in the direction of the neck so as to avoid contamination from the hands. Samples were returned to the laboratory within 2 hr. and 3 loopfuls (4 mm. diam.) were removed and spread on each of 2 nutrient agar plates, which were then incubated at 30° for 3 days. At the end of this period samples from several colonies of each colony type on the isolation plates were inoculated into tubes of peptone water (%, w/v: Oxoid

Table 1. *Cultures received from other workers*

Number	Designation	Donor
NCIB 6750	*Pseudomonas aeruginosa*	National Collection of Indus- trial Bacteria (NCIB)
NCIB 8178	*P. synxantha*	NCIB
NCIB 8295	*P. aeruginosa*	NCIB
NCIB 8297	*Pseudomonas* sp.	NCIB
NCIB 8298	*Pseudomonas* sp.	NCIB
NCIB 8529	*P. hydrophila*	NCIB
NCIB 8530	*P. hydrophila*	NCIB
NCIB 8615	*P. putrefaciens*	NCIB
41	*P. aeruginosa* (?) from human faeces	Dr E. B. Pike
42	*P. aeruginosa* (?) from human faeces	Dr E. B. Pike
78	*P. aeruginosa* (?) from cockroach gut	Mrs E. Healey
79	*P. aeruginosa* (?) from cockroach gut	Mrs E. Healey
80	*P. aeruginosa* (?) from human mouth	Dr A. C. Baird-Parker
81	*P. aeruginosa* (?) from human mouth	Dr A. C. Baird-Parker
99	Gram-negative rod from carious tooth	Dr A. C. Baird-Parker
100	Gram-negative rod from carious tooth	Dr A. C. Baird-Parker

peptone, 1; NaCl, 0·5; in distilled water; pH 7·4) and incubated for 18 hr. In order to have a comparison with bacteria from other habitats, several cultures listed in Table 1 were treated in the same way. In addition 14 Vibrio cultures were studied: the work on these was recorded by Davis & Park (1961). After incubation cultures were examined by the hanging drop method. Those cultures judged by their straight darting movement of the order of 40 μ/sec. to consist of polarly flagellate bacteria were subcultured on nutrient agar to test their purity and then maintained as described by Davis & Park (1961). The flagellation of all the 61 water strains and the 16 strains from other sources was confirmed by electron microscopy. Five cultures which showed zig-zag motion of the order of 10 μ/sec. or less were also retained for electron microscopy.

Determination of morphological cultural and biochemical characteristics

The tests carried out to determine these characteristics were described by Davis & Park (1961).

RESULTS

Flagellation and morphology

All the strains considered to be polarly flagellate because they displayed a straight darting movement were found to have polar flagella and to be Gram-negative. Somatic curvature was a common feature of certain strains of *Pseudomonas fluorescens*, Comamonas and Aeromonas. Neither *S*-forms, spirals or spheroplasts, all considered by Park (1961) and Davis & Park (1961) to be characteristic of members of the genus *Vibrio*, were observed. Cultures of some Comamonas strains included long forms. These were either non-flagellate or had flagella at the poles only. Similar forms, with a different type of flagellation, were reported in Comamonas cultures by Galarneault & Leifson (1956). Of the five strains which showed the kind of motility believed to be associated with peritrichous flagellation (i.e. zig-zag motion of the order of 10 μ/sec. or less) four were found to display typical peritrichous flagellation, while the fifth possessed the flagellation described by Conn & Wolfe (1938) as 'degenerate peritrichous'; this strain had one flagellum arising from some point other than the pole (Pl. 1, fig. 3).

Variations in the number of flagella possessed by organisms of a single culture were observed: a finding similar to those of Bartholomew (1949) and of Rhodes (1958). There was, however, some indication that the degree of variation was reasonably consistent within a group of strains. All of the Comamonas, 12 of the 14 Aeromonas and 9 of the 10 *Pseudomonas aeruginosa* (*P. pyocyanea*) strains consisted chiefly of organisms with a single polar flagellum, though some with 2 polar flagella were also present (Pl. 1, figs. 1, 4). In contrast, 22 of the 30 *P. fluorescens* strains displayed from 1 to 10 flagella/organism (Pl. 1, fig. 7).

Bipolar flagellation was never detected in any of the strains examined. This was in marked contrast to the experience of Park (1961) and Davis & Park (1961) with *Vibrio* spp. and with *V. neocistes* and *V. cyclosites*. However, other workers have reported bipolar flagellation in Pseudomonas (Lehmann & Neuman, 1931; Bisset, 1951; Rhodes, 1958) and in Comamonas (Galarneault & Leifson, 1956).

The flagellation of the two *Aeromonas hydrophila* strains differed markedly from that of other strains classified as *Aeromonas*. Both strains consisted of organisms which had several long thin flagella that appeared to arise from any point on the body (Pl. 1, fig. 6, 8). These strains displayed the kind of motility associated with polarly flagellate bacteria.

Attack on carbohydrates

Certain Comamonas strains brought about a decrease in the pH value of carbohydrate-free peptone water and therefore in most of the carbohydrate-containing peptone water media. However, some strains did not do this in the presence of sucrose or maltose. Acidification of the carbohydrate-free peptone water medium was not brought about by any of the Pseudomonas or Aeromonas cultures. It is obvious that control tubes should be used in carbohydrate tests carried out in peptone water (see Orcutt & Nutting, 1942). No strains acidified the basal medium of

Simon (1956); fermentation results in this medium were the same as those in peptone water.

Four Comamonas strains were able to utilize certain carbohydrates as sole carbon source but they produced no acid from these compounds in complex media. Similar strains were reported by Khambata & Bhat (1953) and by Pivnick (1955).

All but one of the Comamonas strains produced an alkaline reaction in the Hugh & Leifson (1953) open tube. This reaction was restricted to the surface at 24 hr. but it often spread throughout the tube within 7 days. Two of the 4 strains that had been found to utilize glucose and some other carbohydrates as sole carbon source, although they produced an alkaline reaction at the surface of the open tube after 24 hr., showed a reversion to neutral at 7 days. Several Pseudomonas strains produced a little alkali in the open tube within 24 hr. but in every case an acid reaction had developed by 7 days. No changes were observed in the closed tubes inoculated with either Comamonas or Pseudomonas.

Table 2. *Percentage of strains in each genus giving positive results to tests*

Test	Aeromonas 14 strains	Pseudomonas 40 strains	Comamonas 23 strains
Somatic curvature of some organisms	7·1	40·0	13·0
More than 3 flagella/organism	0·0	57·5	0·0
Gas from carbohydrates	92·9	0·0	0·0
Acid in peptone water base + :			
arabinose	92·9	100·0	0·0
glucose	100·0	100·0	0·0
fructose	100·0	20·0	0·0
mannose	100·0	100·0	0·0
galactose	100·0	100·0	0·0
mannitol	100·0	27·5	0·0
dulcitol	7·1	0·0	0·0
sorbitol	50·0	0·0	0·0
sucrose	92·9	17·5	0·0
lactose	42·8	0·0	0·0
maltose	100·0	0·0	0·0
salicin	71·4	0·0	0·0
Utilization as sole carbon source:			
arabinose	92·9	100·0	0·0
glucose	100·0	100·0	17·4
fructose	100·0	20·0	8·7
mannose	100·0	100·0	0·0
galactose	100·0	100·0	17·4
mannitol	100·0	47·5	8·7
dulcitol	0·0	0·0	0·0
sorbitol	50·0	0·0	0·0
sucrose	92·9	17·5	0·0
lactose	42·8	0·0	0·0
maltose	100·0	0·0	0·0
salicin	71·4	0·0	0·0
Fermentation of glucose (Hugh & Leifson)	100·0	0·0	0·0
Oxidation of glucose (Hugh & Leifson)	?	100·0	0·0
Reducing compounds from gluconate	79·0	92·5	0·0
Acetoin production	92·9	0·0	0·0
Methyl red test	0·0	0·0	0·0
Modified Eijkman test	0·0	0·0	0·0

Table 2 (*cont.*)

Test	*Aeromonas* 14 strains	*Pseudomonas* 40 strains	*Comamonas* 23 strains
Starch hydrolysis	79·0	30·0 (W)	0·0
Nitrate reduction	100·0	32·5	47·8
Cholera red reaction	0·0	0·0	0·0
Indole production	71·4	0·0	0·0
Utilization as sole carbon source of:			
tryptophan	7·1	57·5	13·0
phenylalanine	0·0	95·0	17·4
phenol	0·0	20·0	0·0
benzoic acid	7·1	72·5	0·0
catechol	7·1	47·5	0·0
Attack on:			
tyrosine	64·3	97·5	87·0
phenylalanine (Shaw & Clarke)	0·0	10·0	4·4
malonate (Shaw & Clarke)	71·4	100·0	56·5
gelatin	100·0	72·5	39·1
serum	64·3	27·5	0·0
casein	57·1	27·5	17·4
H_2S from peptone	100·0	22·5	52·2
Urea hydrolysis	21·4	67·5	4·4
Tributyrin hydrolysis	100·0	100·0	100·0
Utilization as sole carbon source of:			
succinate	100·0	100·0	69·6
malate	100·0	100·0	60·9
acetate	92·9	100·0	65·2
citrate	100·0	100·0	60·9
pyruvate	92·9	100·0	69·6
lactate	28·6	100·0	69·6
malonate	21·4	100·0	34·8
formate	7·1	90·0	43·4
ethanol	0·0	90·0	39·1
Catalase production	100·0	100·0	100·0
Kovacs oxidase test	57·1	90·0	56·5
Cytochrome oxidase test	57·1 (L)	90·0 (+ & L)	56·5 (+ & L)
Growth at:			
0°	100·0	52·5	30·4
37°	92·9	80·0	95·6
42°	21·4	25·0	34·8
45°	0·0	25·0	13·0
Growth in presence of:			
1 % (w/v) NaCl	100·0	100·0	100·0
3 % (w/v) NaCl	71·4	95·0	91·3
6 % (w/v) NaCl	14·3	25·0	21·7
9 % (w/v) NaCl	0·0	0·0	4·4
Growth on:			
MacConkey's agar	85·7	77·5	73·9
Aronson's agar	28·6	0·0	52·2
Production of u.v.-fluorescent compounds	0·0	100·0	0·0
Sensitivity to:			
penicillin (2·5 i.u.)	0·0	0·0	0·0
terramycin (10 μg.)	100·0	52·5	100·0
streptomycin (80 μg.)	100·0	100·0	78·3
chloramphenicol (100 μg.)	100·0	80·0	95·6
compound 0/129	0·0	0·0	0·0

W = weak activity; L = delayed reaction.

Utilization of ethanol

Utilization of ethanol as a sole carbon source was a property displayed by all but 4 of the Pseudomonas strains studied. All the *Pseudomonas aeruginosa* strains and 7 of the *P. fluorescens* strains brought about a clearing of the chalk medium, indicating that excess acid had been produced. Nine Comamonas strains utilized ethanol, 2 producing excess acid. None of the Aeromonas strains were active in this respect. It should be emphasized that this test is not the same as the 'acid in ethanol + CaCO₃ agar' test of Shimwell, Carr & Rhodes (1960) whose medium, which was recommended for the differentiation of *Acetomonas* and *Pseudomonas*, contains a higher concentration of ethanol and CaCO₃ and also 1 % (w/v) yeast extract.

Production of fluorescent compounds

Four of the strains identified as Pseudomonas were never seen to produce fluorescin. However, these 4 and all the other members of this genus, but no other strains, produced compounds which fluoresced under a source of ultraviolet radiations (u.v.) when they were grown on glucose + chalk medium or in the medium of Georgia & Poe (1931).

Details of results

The complete details of results are not given here; those interested may obtain them from the author. The percentage of strains in each group which reacted positively to the various tests is given in Table 2.

DISCUSSION

All the 77 polarly-flagellate rod-shaped bacteria examined in this study were classifiable into one of three main groupings. While it is difficult, if not impossible, to define each of these groups in an exclusive manner, as is probably the case with all groups of bacteria (Sneath, 1957), the author feels that the characters of each of these groups given below are sufficient to allow their differentiation in practice.

Aeromonas. Fermentative attack on carbohydrates shown in Hugh & Leifson's medium. No pigments or u.v.-fluorescent compounds produced.

Pseudomonas. Oxidative attack on carbohydrates displayed in Hugh & Leifson's medium. Diffusible compounds that fluoresce under u.v. radiation produced on glucose + chalk medium, in the medium of Georgia & Poe and on several other media. Pigments visible in daylight are not produced by all strains.

Comamonas. No attack on carbohydrates shown in complex media. Usually unable to utilize carbohydrates as sole carbon sources. No pigments or u.v.-fluorescent compounds produced.

The distribution between the three genera of other features can be seen in Table 2.

Aeromonas

Eleven of the 14 strains included in Aeromonas were almost identical. The two type strains of *A. hydrophila* and strain 137 (author's no.) were exceptions. None

of the Aeromonas strains isolated from water were seen to have other than true polar flagellation, in contrast to both strains of *A. hydrophila* (see Results). Miles & Halnan (1937) and Kulp & Borden (1942) reported that Aeromonas organisms had only one polar flagellum, but Miles & Miles (1951) found that some had as many as eight. Leifson & Hugh (1953) reported that in young cultures peritrichously flagellate organisms were often present. Strain 137 differed from the other Aeromonas strains in not producing gas during carbohydrate fermentation. It also differed in being Voges–Proskauer negative and in utilizing various aromatic compounds as sole carbon source. The strain was very similar to *A. formicans* described by Crawford (1954) and by Pivnick & Sabina (1957). It was, however, methyl red negative, incapable of growth at 42° and it fermented arabinose. A strain similar to no. 137 was isolated from tap water on Dieudonné's medium by Dr G. H. G. Davis (personal communication).

Several fermentative rod-shaped bacteria with polar flagella have been described and it is possible that even more kinds might have been, had flagellar pattern determination been a simpler process than it is. Following Kluyver & van Niel (1936) the strains producing 2:3 butylene glycol as an end-product of glucose fermentation and therefore also giving a positive Voges–Proskauer reaction are known as Aeromonas, while those known to ferment carbohydrates with the production of ethanol are placed in a separate genus, *Zymomonas*. Strains that showed a typical *Escherichia coli* fermentation except for the fact that gases were not produced were classified as *Aeromonas* by Pivnick & Sabina (1957) since these authors considered that all fermentative pseudomonads except *Zymomonas* should, temporarily at least, be included in a single genus. The present author feels that it would be more satisfactory to include all polarly flagellate rod-shaped fermentative bacteria in a single genus, *Aeromonas*, with specific rank being given to strains which show different end-products of fermentation. He considers that the ability to ferment carbohydrates is a more basic characteristic than is the production of a particular end-product of fermentation. Stevenson's (1959) suggestion that Aeromonas strains are in reality non-pigmented *Serratia* has been shown to be not acceptable (Eddy, 1960; Liu, 1961a). For a more detailed discussion of the genus *Aeromonas* the reader is referred to Eddy (1960).

When only cultural and biochemical characters are studied there is a risk of classifying Aeromonas bacteria as members of the Enterobacteriaceae (Crawford, 1954; Eddy, 1960; Ewing & Johnson, 1960). Certainly one cannot rely on the test used by Thomas, Hobson & Druce (1959) and others (i.e. the ability to produce acid and gas in lactose MacConkey broth within 5 days at 30°) to diagnose coli-aerogenes bacteria, since Aeromonas strains which react positively in the test are known (Eddy & Kitchell, 1959). In fact, in the present state of our knowledge it seems wise to determine the flagellar pattern of any Gram-negative rod that is being classified. Because the determination of flagellar pattern is difficult and time-consuming, and because the classification of non-flagellate forms and forms which show a variation in the type of flagellation they display (Leifson & Hugh, 1953; Sneath, 1956; Adams, Williams & Payne, 1961) is not helped by such a determination, biochemical tests that will enable one to separate the polarly flagellate group from the peritrichously flagellate group are needed. The tests concerned with arginine metabolism (Sherris, Shoesmith, Parker & Breckon, 1959; Thornley, 1960), and the

modified cytochrome oxidase test (Ewing & Johnson, 1960), in which only polarly flagellate rods give positive results, are promising in this respect. It should be noted that the Aeromonas strains studied in the present work gave late or negative results in the original form of the cytochrome oxidase test.

It would seem to be a logical step to unite *Vibrio comma* and closely related organisms (Davis & Park, 1961) with *Aeromonas* spp. in a single genus, but the distinctive morphology that *Vibrio* species display and the absence of cross reactions between the soluble antigens of the two genera (Caselitz, 1960) makes the author hesitate to do this; others may be bolder. Certain Aeromonas strains are capable of growing on media such as Aronson agar and Dieudonné medium, usually considered to be selective for *Vibrio*, and some are sensitive to the phosphate derivative of the vibriostatic compound described by Shewan et al. (1954) and Rhodes (1959). Somatic curvature and also fermentation of carbohydrates without gas production, both sometimes considered to be diagnostic of *Vibrio*, are features of some Aeromonas strains. In view of the similarities between the two genera, one wonders whether some of the 'vibrios' studied by Taylor, Pandit & Read (1937), Jackson (1948) and others were *Aeromonas* spp. It appears that the methods used for isolating and identifying the 'vibrios' would not have excluded *Aeromonas*. It is, in fact, difficult to define *Aeromonas* and *Vibrio* in such a way as to make them mutually exclusive, as will be obvious from a comparison of the results reported here for *Aeromonas* with those reported by Davis & Park (1961) for *Vibrio*.

Note. Dr B. P. Eddy of the Low Temperature Research Station, Cambridge, and Dr K. J. Steel of the National Collection of type cultures have drawn the author's attention to the work of Clement & Gibbons (1960). Clement and Gibbons (1960) reclassified *Aeromonas hydrophila* strains NCIB 8529 and NCIB 8530 as *Aerobacter cloacae* because the organisms were peritrichate and differed from 'authentic' Aeromonas in several biochemical characters. The fact that the kind of flagellation displayed by these strains was not typical of Aeromonas is also recorded in the present paper. The strains were retained for study because many organisms that were possibly polarly flagellate were observed (e.g. Pl. 1, fig. 8). It was thought that strains 8529 and 8530 were showing a variation in flagellar pattern similar to that reported by Leifson & Hugh (1953) to occur in several members of the genus *Aeromonas* including one of the type cultures of *A. hydrophila* (i.e. ATCC 7965) considered by Clement & Gibbons (1960) to be authentic. While the work of Clement & Gibbons (1960) shows that strains 8529 and 8530 are more satisfactorily classified as *Aerobacter cloacae* than as *Aeromonas hydrophila*, it should be remembered that several of the biochemical characters are said by these authors to differentiate 8529 and 8530 from 'authentic' Aeromonas known to be possessed by some polarly flagellate fermentative bacteria (see Eddy, 1960; Park, present paper).

Pseudomonas

The possession by a polarly flagellate Gram-negative rod of the ability to oxidize carbohydrates and/or to produce compounds that fluoresce under u.v. radiation is thought by the author to be sufficient to justify its classification as *Pseudomonas*. Forty strains studied in this work had both these characteristics; none was found with one but not the other character. Thornley (1960), who examined oxidative,

polarly flagellate rods for their ability to produce u.v.-fluorescent compounds on a proteose-peptone + glycerol + salts medium, found that only about one-third of her strains had this ability. It is not known whether the results obtained on the medium she used are strictly comparable with those obtained on glucose + chalk medium used in the present studies, but it does appear that completely non-u.v.-fluorescent Pseudomonas do occur. Neither *Bergey's Manual* (1957) nor Sherris *et al.* (1959) considered that pigment production should be relied upon for identifying Pseudomonas bacteria. However the presence of a green diffusible fluorescent pigment in cultures of polarly flagellate Gram-negative rods is generally regarded as being indicative of *Pseudomonas*. Although Rhodes (1959) used this character in isolating her Pseudomonas strains, she concluded because of reports of production of fluorescent pigments, not fluorescin, by Azotobacter (Johnstone, 1955) and *Bacillus subtilis* (Shank, Chmura & Silliker, 1958) that this character had no diagnostic value. The present author thinks that the ability to produce u.v.-fluorescent compounds is of diagnostic value when positive, if used in conjunction with other characters such as polar flagellation.

Other reactions given by the Pseudomonas strains studied are shown in Table 2. Generally these agree with those given for Pseudomonas by Seleen & Stark (1943) Rhodes (1959) and others. The ability to utilize various aromatic compounds as sole carbon source is a property that the author has found to be common in the genus.

The arginine tests described by Sherris *et al.* (1959) and by Thornley (1960) were not used with the organisms studied here. It seems that these tests are of considerable value in distinguishing between *Pseudomonas* and peritrichously or non-flagellate bacteria, but of less value in separating *Pseudomonas* from other polarly flagellate bacteria between which there appear to be only quantitative differences.

The separation of *Pseudomonas aeruginosa* from other members of the genus on the basis of temperature requirements for growth and on the production of pyocyanine now seems well established (Seleen & Stark, 1943; Haynes, 1951). All the Pseudomonas strains studied here that were capable of growth at 42° also produced pyocyanine and these were classified as *P. aeruginosa*. As can be seen from Table 3, certain other properties were more common in this species than in the *P. fluorescens* strains studied. No *P. aeruginosa* strains were isolated from water. The gluconate test of Haynes (1951), the oxidase test of Kovacs (1956) and the cytochrome oxidase test of Gaby & Hadley (1957), all of which were developed to distinguish *P. aeruginosa* from other Gram-negative bacteria found in clinical material, gave positive results with some *P. fluorescens* strains also. Although the two species show many common characteristics (see also Rhodes, 1959), as one would hope, since they are classified in the same genus, there appears to be little chance of confusing them, even if pyocyanine production is ignored. The studies of Liu (1961b) on the serology of extracellular antigens produced by various Pseudomonas strains, in which it was found that *P. aeruginosa* strains had a species specific antigen, confirmed that the two species are distinct.

The success in separating *Pseudomonas aeruginosa* from *P. fluorescens* on the basis of the upper temperature limit for growth made it tempting to subdivide *P. fluorescens* on the ability to grow at 0°. While there was no other characteristic correlating with growth at 0°, separation on this basis led to two groups which showed marked differences in the distribution of positive results to various tests. It is

appreciated that the number of strains involved is small and the results possibly not significant. The separation is mentioned in the hope that others will carry out tests to determine the ability of their *P. fluorescens* strains to grow at 0°, to utilize phenol when supplied at 0·05 % (w/v), and to utilize catechol and benzoic acid. Perhaps, as Jakoby, Schatz, Hutner & Weber (1952) suggested, pseudomonads can be classified on their ability to utilize various aromatic compounds.

Table 3. *Percentage of strains in each of three groups of* Pseudomonas
that gave positive results in various tests

Test	*P. aeruginosa* 10 strains	*P. fluorescens* (1) 21 strains	*P. fluorescens* (2) 9 strains
Pyocyanine production	100·0	0·0	0·0
Growth at 42°	100·0	0·0	0·0
Growth at 0°	0·0	100·0	0·0
Growth in 6 % (w/v) NaCl	100·0	0·0	0·0
Utilization of:			
fructose	0·0	33·3	11·1
sucrose	0·0	33·3	0·0
mannitol	80·0	52·4	0·0
tryptophan	90·0	57·1	22·2
benzoate	100·0	47·6	100·0
catechol	90·0	4·8	100·0
phenol	0·0	0·0	88·8
Attack on:			
starch	0·0	47·6	22·2
gelatin	100·0	90·5	0·0
casein	100·0	4·8	0·0
serum	90·0	9·5	0·0
Reduction of nitrate	90·0	19·1	0·0
More than 3 flagella/organism	10·0	71·4	77·8

Comamonas

Several of the organisms studied here gave negative results in most of the tests used, and because of this they were considered to be members of a single group. It is thought that the group is sufficiently distinct from either *Aeromonas* or *Pseudomonas* to merit generic rank. It is not possible to decide on present evidence whether one or more species is represented. There is, however, a gradation in characteristics shown by members of this genus that at the more active extreme suggests a relationship with *Pseudomonas* (see also Davis & Park, 1961). Four strains studied (author's nos. 32, 33, 34, 36) were most striking in this respect. They did not produce excess acid from carbohydrates supplied in complex media, either because other compounds were utilized preferentially or because there were no acidic end products of carbohydrate attack, but they were capable of utilizing some carbohydrates as sole carbon source. They also showed an ability to utilize a number of other organic compounds as sole carbon sources.

The Comamonas strains studied all possessed one or occasionally 2 flagella at one pole and in this way they differed from *Lophomonas* (Galarneault & Leifson, 1956) which was described as having a tuft of from 2 to 4 flagella at one or both poles. There is a need to classify polarly flagellate strains which resemble Alcaligenes in giving negative reactions to many biochemical tests, separately from other morphologically similar types. Lehmann & Neumann (1931) included such forms in

a single species, *Vibrio alcaligenes*; but this is not a satisfactory arrangement since they differ from the type species, *V. comma*, both in their morphology and physiology, (see Davis & Park, 1961). Galarneault & Leifson (1956) intended the genus *Lophomonas* for those bacteria physiologically like *Alcaligenes* that had more than one polar flagellum, monotrichous forms being retained in *Pseudomonas* (Leifson, 1958). They claimed that *L. alcaligenes* was synonymous with *V. alcaligenes* (Lehmann & Neumann) but this was not so since the latter species was originally defined as consisting of organisms having from 1 to 6 polar flagella. Davis & Park (1961) pointed out that the name *Lophomonas* is invalid and they substituted the name *Comamonas*. The author can see no reason for excluding those bacteria of the Alcaligenes type having predominantly only one polar flagellum from similar but lophotrichous forms and he therefore considers that the genus *Comamonas* should include all those polarly flagellate Gram-negative rods physiologically similar to *Alcaligenes* that do not display a typical *Vibrio* morphology (Park, 1961; Davis & Park, 1961).

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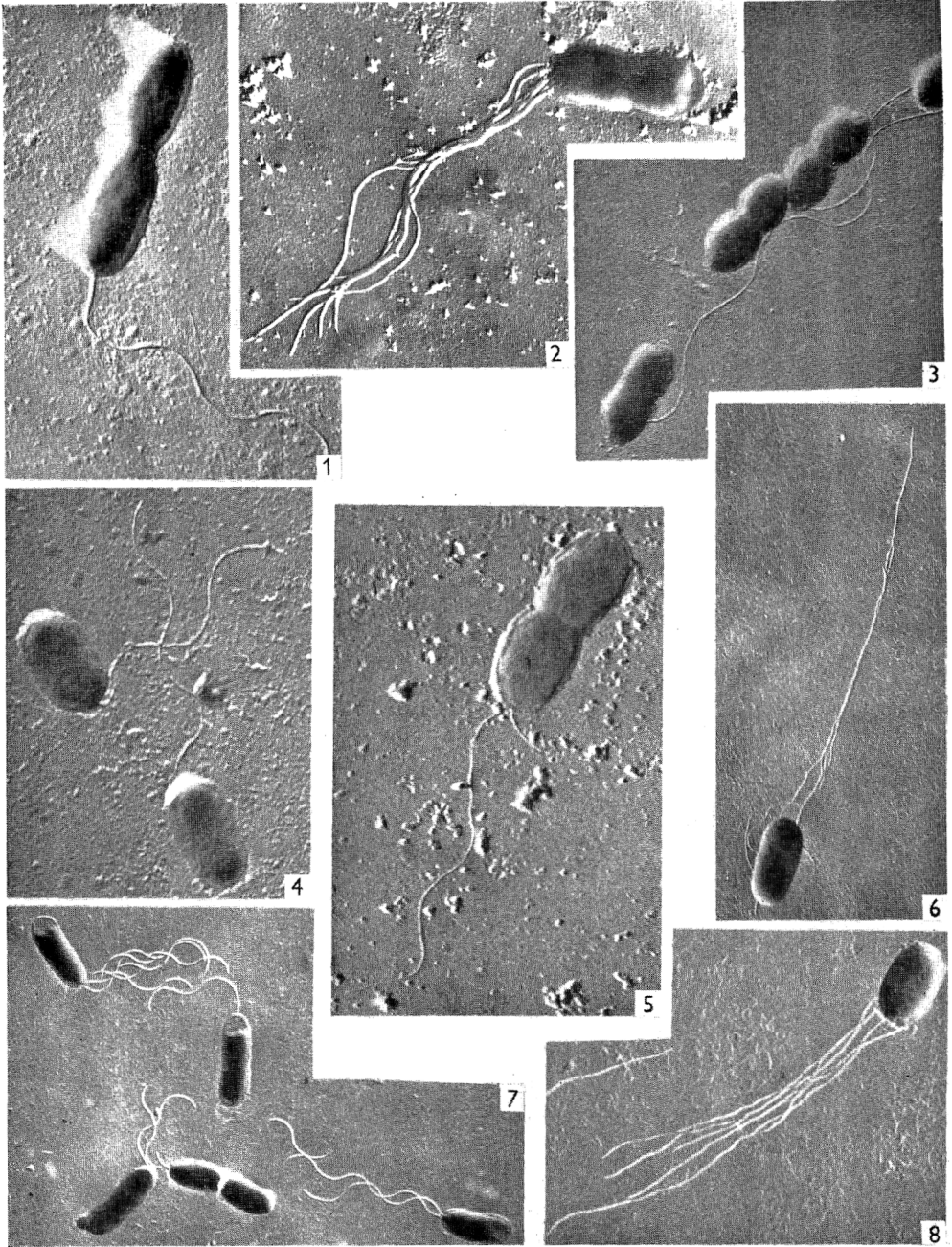
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EXPLANATION OF PLATE

All figures are electron micrographs of gold-palladium shadowed specimens.

Fig. 1. *Aeromonas* sp. (strain 1). The organism is dividing and has a flagellum at one pole only. × 15,500.

Fig. 2. *Pseudomonas aeruginosa* (strain 42). The organism has many flagella at one pole and none at the other. It is dividing. × 10,500.

Fig. 3. Organisms of a strain that was not studied in detail showing degenerate peritrichous flagellation. × 8,500.

Fig. 4. *P. aeruginosa* (strain 81). Organisms with single polar flagella. × 10,500.

Fig. 5. *P. fluorescens* (strain 71). A dividing organism with flagella at one pole only. × 10,500.

Figs. 6, 8. *A. hydrophila*. Two of the kinds of flagellar arrangement observed in the cultures examined.

Fig. 6. *A. hydrophila* (NCIB 8529). × 6,500.   Fig. 8. *A. hydrophila* (NCIB 8530). × 9,500.

Fig. 7. *P. fluorescens* (strain 112). Showing variation in the number of flagella possessed by different organisms in the same culture. × 5,000.

Metabolic Patterns in Acetic Acid Bacteria

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SUMMARY

Evidence is presented that the distinction, already made on nutritional grounds, between lactaphilic and glycophilic strains of acetic acid bacteria may be traced back to the metabolic level. Thus, the capacity of cell extracts to effect reversible transamination from glutamate to aspartate was well developed in lactaphiles, but only feebly so, or not at all, in glycophiles. Again, suspensions of lactaphiles possessed greater general ability to oxidize intermediates of the tricarboxylic acid cycle than did those of glycophiles. Cell extracts of lactaphiles possessed citrogenase activity, which was not detected in glycophiles. The conversion of L-aspartate to α-alanine by extracts of lactaphiles appeared to proceed by β-decarboxylation, not by transamination.

INTRODUCTION

Previous work (Rainbow & Mitson, 1953; Brown & Rainbow, 1956) indicated that acetic acid bacteria might be grouped, on nutritional and biochemical grounds, into lactaphiles and glycophiles. In seeking the metabolic basis of the observed differences, three aspects of the metabolism of five lactaphilic and four glycophilic strains of *Acetobacter* were studied: (*a*) the capacity of cell extracts to effect transaminations involving glutamate, aspartate and α-alanine; (*b*) the capacity of suspensions of whole organisms to oxidize intermediates of the tricarboxylic acid (TCA) cycle; (*c*) the citrogenase activity of cell extracts.

METHODS

Materials

Substrates were commercial preparations of laboratory reagent grade, except succinic acid and sodium acetate, which were of analytical reagent grade. No ninhydrin-reacting impurities were detected when 180 μg. portions of the amino acids were chromatographed on Whatman No. 4 paper.

Test organisms and cultivation

Stock cultures of the test organisms were maintained as described by Rainbow & Mitson (1953). The organisms were the strains of Acetobacter studied by those authors and the strains of *Acetobacter ascendens* and *A. oxydans* studied by Brown & Rainbow (1956). However, the culture previously called *A. suboxydans* NCIB 7069 is referred to in this present paper as *A. rancens* which we now believe to be its correct name. Brown & Rainbow (1956) drew attention to the anomalous position of their *A. suboxydans* strains as lactaphiles. Later, in a private communication to

one of us (C. R.) Dr J. D. Shimwell (British Vinegars Ltd., Frome) pointed out that authentic cultures of *A. suboxydans* by definition do not oxidize acetate, and that the strains reported on from this laboratory might be wrongly labelled. This proved to be the case, for Dr Shimwell, who kindly examined our strains, reported that three strains labelled *A. suboxydans* (including NCIB 7069) were vigorous over-oxidizing *Acetobacter* spp.

For manometric work with suspensions of organisms, these were grown as described by Brown & Rainbow (1956) except that the glycophiles were grown on the same lactate-containing medium as were the lactaphiles, in order to ensure as far as possible that differences in the enzyme make-up of the organisms were not ascribable to cultivation in different media. Organisms were harvested (centrifuge) after incubation for 3–5 days, washed three times (aseptic precautions) with 25 ml. portions of 0·85 % (w/v) NaCl and finally suspended in 0·05 M-phosphate buffer (pH 5·8). Optical densities of these suspensions were determined turbidimetrically by the Spekker photoelectric absorptiometer (Hilger, London) and the values thus obtained were related to cell dry matter by reference to curves previously constructed for each strain of Acetobacter. The suspensions were then diluted with buffer to an appropriate standard of cell dry matter.

For the preparation of cell extracts, organisms were grown and harvested as above, except that they were washed in 1·1 % (w/v) KCl solution, and they were finally suspended in about 8 ml. of ice-cold 0·1 M-phosphate buffer (pH 5·8) before being disrupted.

Escherichia coli ATCC 4157 organisms, required as a source of coenzyme A and transacetylase in citrogenase experiments, were grown as described by Ochoa, Stern & Schneider (1951). They were harvested and washed as described above.

Experiments with suspensions of organisms

Manometric measurements of the oxygen taken up at 35° by washed whole organisms during the oxidation of organic acids were carried out by a conventional technique with the Warburg constant-volume respirometer.

Experiments with cell extracts

Cell extracts. These were prepared from the suspensions of acetobacters and *Escherichia coli* ATCC 4157 in phosphate buffer (see above) by mechanical disintegration with ballotini beads (0·2–0·3 mm. diam.) for 30 min. under refrigerated conditions in a tissue disintegrator (H. Mickle, Gomshall, Surrey). After disruption, the suspension was washed from the beads with small portions (about 10 ml. in all) of ice-cold 0·05 M-phosphate buffer (pH 7·3). The resulting suspension was centrifuged at 0–2° for 20 min. at 20,000 *g* to yield a residue possessing little or no relevant enzymic activity, and a 'soluble enzyme' fraction. The latter was used for transaminase, aspartate decarboxylase and citrogenase experiments. The total nitrogen of this fraction was determined by the micro-Kjeldahl procedure, or its protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Transaminase and β-decarboxylase activities. Evidence of these was sought through the production of new ninhydrin-reacting substances revealed by paper chromatography. In addition, aspartate β-decarboxylase activity was followed by con-

ventional Warburg manometric measurement of the CO_2 released at 25° by cell extracts acting on L-aspartic acid at pH 5·2.

Citrogenase activity. This was tested by a slight modification of the procedure of Ochoa *et al.* (1951) which depends on the formation of citrate from acetyl phosphate and oxaloacetate in the presence of citrogenase, coenzyme A and transacetylase, the two last named being supplied as a cell extract of *Escherichia coli* ATCC 4157, which is itself deficient in citrogenase. Thus, those extracts of acetobacters capable of catalysing citrate synthesis from acetyl phosphate, oxaloacetate and the extract of *E. coli* presumably did so by virtue of their citrogenase content. Under the conditions of the assay there was a linear relationship between enzyme concentration and citrate produced. Citrate was determined colorimetrically by the method of Macdonald & Waterbury (1959). In our hands, there was a linear relationship between optical density at 445 mμ. and citrate content up to at least 100 μg.

Chromatography

Whatman No. 4 paper was used in conventional unidimensional descending or ascending technique.

For descending runs in transaminase experiments, two developing solvents were used: (*a*) *n*-butanol + acetic acid + water (25 + 6 + 25, v/v); (*b*) phenol saturated with water. For the aspartic β-decarboxylase experiments, descending runs in solvent (*a*) and ascending runs in ethanol + ammonia (sp. gr. 0·880) + water (90 + 5 + 5, v/v) were applied. Amino acids were detected by dipping the papers in a solution of 0·1 % ninhydrin in acetone containing 1 % (w/v) of 2:4:6:-collidine and then heating at 60° for 15 min. After storing these papers in the dark overnight, the presence of 2-oxoglutarate was revealed as a spot which fluoresced in the light of a mercury vapour lamp (Rabson & Tolbert, 1958). Oxaloacetate could not be distinguished from pyruvate by this method.

Chromatographic identification of an unknown compound was presumed when (i) both the unknown and an authentic sample had the same chromatographic mobilities in both solvents; (ii) one spot of appropriately increased size and intensity was obtained when the unknown was chromatographed in the presence of authentic sample.

RESULTS

Transaminase activities

The ability of cell extracts of acetic acid bacteria to effect reversible transaminations in the following systems was tested:

    (*a*) L-glutamate + oxaloacetate $\rightleftharpoons$ 2-oxoglutarate + L-aspartate;

    (*b*) L-glutamate + pyruvate $\rightleftharpoons$ 2-oxoglutarate + L-alanine;

    (*c*) L-aspartate + pyruvate $\rightleftharpoons$ oxaloacetate + L-alanine.

The results (Table 1) showed that, in 3 hr., extracts of all lactaphiles effected reversible transamination as in (i). By contrast, extracts of glycophiles showed an undetectable effect (*Acetobacter gluconicum*) or only a small effect (*A. capsulatum, A. turbidans, A. viscosum*), the amount of transamination brought about even when incubation was prolonged for 24 hr. being much less than that brought about by the lactaphiles in 3 hr. There was no clear evidence of transaminations proceeding as in

(b) and (c) at pH values between 5·6 and 9·0 In those cases (*Acetobacter acidum-mucosum, A. rancens, A. oxydans*) where more than traces of α-alanine were produced from aspartate and pyruvate, the reverse formation of aspartate from oxaloacetate and α-alanine did not occur, and α-alanine was formed from aspartate even in the absence of added pyruvate. This indicated that α-alanine formation from aspartate involved a mechanism other than transamination, possibly β-decarboxylation (see below).

Table 1. *Transaminative abilities of cell extracts of acetic acid bacteria*

Reaction mixtures (1 ml.) contained cell extract (equiv. 35 μg. total-N) and substrate solution at pH 7·3. Substrate solutions contained 20 μmole of L-glutamate or L-aspartate or 40 μmole of DL-alanine alone or in admixture with 20 μmole of oxaloacetate, 2-oxoglutarate or pyruvate. Transformation products (amino acid, 2-oxoglutarate) were detected by paper chromatography of samples (30 μl.) withdrawn after 3 hr. incubation at 28°.

	Ninhydrin-reacting substances produced from						
Acetobacter spp.	glu + oac	asp + ogl	glu + pyr	ala + ogl	asp + pyr	ala + oac	asp
Lactaphiles:							
A. acidum-mucosum	qsp ogl (ala)	glu ala	(ala)	nil	ala	nil	ala
A. mobile	asp (ala)	glu (ala)	nil	nil	(ala)	nil	(ala)
A. rancens	asp ogl (ala)	glu ala	(ala)	nil	ala	nil	ala
A. ascendens	asp ogl (ala)	glu (ala)	(ala)	nil	nil	nil	(ala)
A. oxydans	asp	glu ala	nil	nil	ala	nil	ala
Glycophiles:							
A. capsulatum	(asp)	(glu)	nil	nil	nil	nil	nil
A. gluconicum	nil	nil	nil	nil	nil	nil	nil
A. turbidans	(asp)	(glu)	nil	nil	nil	nil	nil
A. viscosum	(asp)	(glu)	(ala)	nil	nil	nil	nil

ala, α-alanine; asp, aspartate; glu, glutamate; oac, oxaloacetate; ogl, oxoglutarate; pyr, pyruvate, ( ) indicates trace amounts.

With glutamate + oxaloacetate as substrate in a system catalysed by an extract of *Acetobacter mobile*, chromatography showed that aspartate formation occurred within the range of pH 5·3–7·8. At pH 7·3 (approximately the optimum) transamination occurred readily at temperatures from 21–28°: there was only a little action in 4 hr. at 37° and none at 47°.

Simple dialysis of the 'soluble enzyme' preparation from *Acetobacter mobile* for 50 hr. at 2° against 0·01M-phosphate buffer (pH 7·3) failed to inactivate the preparation catalysing system (i). However, its capacity to effect this reversible transamination was greatly diminished by ultrafiltration (Grant, Rowe & Stanworth, 1958). The enzyme thus treated was reactivated by addition of the dialysate or of 1·7 μg. pyridoxal phosphate/ml. When stored at −15°, cell extracts at pH 7 retained their transaminative ability substantially undiminished for periods of many months.

Aspartate β-decarboxylase activities

The formation from L-aspartate of appreciable quantities of a ninhydrin-reacting material having the chromatographic mobility of α-alanine indicated that cell extracts of the lactaphiles possessed an aspartate decarboxylase. Of the glycophiles, only *Acetobacter viscosum* possessed this system, and that only to a small extent.

Freshly prepared cell extracts of *Acetobacter acidum-mucosum*, which were particularly potent in ability to attack aspartate, were selected for further study. Manometric measurements (Table 2) showed that such extracts released an alkali-soluble gas from L-aspartate solutions at pH 5·2. The evolution of gas was approximately linear over a period from 5–20 min., when the amount of gas evolved was nearly 70 % of that theoretically obtainable from the aspartate added. After standing at laboratory temperature for a further hour, the reaction mixtures in the Warburg vessels were examined by paper chromatography. The ethanol+ammonia +water solvent in particular permitted adequate resolution of aspartate, α-alanine

Table 2. *Decarboxylation of* L-*aspartate by cell extracts of*
Acetobacter acidum-mucosum

Each Warburg vessel contained 3 ml. total volume. Complete reaction mixtures contained L-aspartic acid, adjusted to pH 5·2 (8 μmole); acetate buffer, pH 5·2 (0·3 m-mole); and enzyme (1·6 mg. protein, added from side-arm). The centre well contained 0·2 ml. 20 % KOH only where indicated. Under the conditions used, the yield of CO_2 theoretically obtainable by β-decarboxylation of 8 μmole L-aspartate was 195 μl.

	μl. CO_2 evolved at 25° in			
Reaction mixture	5 min.	10 min.	15 min.	20 min.
No enzyme	6	8	8	8
No aspartate	7	8	9	10
Complete	20	60	97	134
Complete (KOH in centre well)	9	9	9	9

and β-alanine, which were also distinct in colour of their ninhydrin compounds (α-alanine, purple; β-alanine, blue) after runs in this solvent. The results showed that (i) virtually all the aspartate had disappeared from the reaction mixtures containing initially both enzyme and aspartate; (ii) a spot of appropriately increased size was obtained when the reaction mixture was supplemented with α-alanine, whereas supplementation with β-alanine gave a second spot, distinct in mobility in both solvents and in colour in the ethanol+ammonia+water solvent; (iii) the cell extract alone contributed no appreciable quantities of amino acids to the reaction mixtures. Thus, cell extracts of *Acetobacter acidum-mucosum* catalysed the β-decarboxylation of L-aspartate to α-alanine; β-alanine, the product of α-decarboxylation, was not produced. The aspartate β-decarboxylase activity of cell extracts of *A. acidum-mucosum* was retained on storage at −15° for at least one month.

Oxidation of organic acids

Oxidation of organic acids was studied manometrically with whole organisms. On adding substrate from the side-arm, little or no oxygen was absorbed during the first 5–10 min., but thereafter there was a linear rate of oxygen uptake from which Q_{O_2} values were calculated. The results (Table 3) show that the lactaphiles had

greater general ability to oxidize organic acids than had the glycophiles, the oxidative abilities of which were restricted mainly to lactate, pyruvate and oxaloacetate, and were relatively feeble or lacking with respect to all other substrates. In a few cases, restricted entirely to the lactaphiles, appreciable stimulations of oxygen uptake were recorded when a mixture of 0·03 μmole of each of the following was added to the main substrate undergoing oxidation in the Warburg vessel: citric, isocitric, cis-aconitic, 2-oxoglutaric, succinic, fumaric, malic and oxaloacetic acids.

Table 3. *Oxidative abilities of washed suspension of acetic acid bacteria acting on organic acids*

Warburg vessels contained in 2·2 ml. final volume: washed organisms equiv. 0·2–0·3 mg. dry wt.; phosphate buffer (pH 5·8; 50 μmole); substrate (pH 5·8; 100 μmole added from side-arm); 0·2 ml. 20% KOH (centre well). Temperature 35°. The values have been corrected for oxygen uptake in the absence of the major substrate. Values in parentheses indicate oxygen uptakes in the presence of 'sparker' amounts of mixed TCA cycle intermediates in those cases where oxidation was appreciably stimulated.

Acetobacter spp.

Substrate	acidum-muco-sum	mobile	rancens	ascen-dens	oxydans	capsu-latum	gluco-nicum	turbi-dans	visco-sum
					Q_{O_2} values				
Lactate	66	336	763	280	288	93	10	158	64
Pyruvate	97	150	400	64	74	82	15	40	157
Acetate	0	58	24	5	16	0	6	0	0
		(112)			(35)				
Oxaloacetate	198	167	270	228	254	220	19	25	41
Citrate	20	115	102	226	42	0	0	16	0
Isocitrate	39	19	3	4	17	0	0	3	18
			(18)		(42)				
Cis-aconitate	15	44	25	93	48	0	0	0	11
2-Oxoglutarate	25	46	8	95	48	0	0	0	10
Succinate	68	155	100	345	143	0	0	21	7
Fumarate	0	22	22	150	101	0	20	7	0
L-Malate	20	108	39	120	104	0	5	24	0

Citrogenase activities

The general ability of lactaphiles, but not of glycophiles, to oxidize intermediates of the tricarboxylic acid (TCA) cycle indicates that a TCA cycle may operate in lactaphiles but not in glycophiles. This suggestion was already implicit in the work of Brown & Rainbow (1956), who showed that lactaphiles, but not glycophiles, could grow on acetate and corresponded to Vaughn's (1942) 'over-oxidizers', which oxidized acetate to CO_2 and water. It seemed therefore desirable to examine acetobacters for citrogenase, the enzyme which enables acetate to enter the TCA cycle. The results (Table 4) showed that extracts from each of the lactaphiles catalysed the synthesis of citrate from acetyl phosphate and oxaloacetate. Extracts of the glycophiles apparently did not, the blank readings being greater than those given by the test reaction mixtures. There is reason to believe that this effect resulted from enzymic decarboxylation of oxalaocetate, which is the constituent mainly responsible for the colour developed in the blanks in the reaction applied to determine citrate. Although oxaloacetic decarboxylase activity could be detected

in extracts of glycophilic and lactaphilic organisms (P. J. le B. Williams, unpublished observations), its effect on the citrate colour reaction, detectable in the absence of appreciable citrate synthesis, would be swamped in the citrogenase tests involving extracts of lactaphilic cells, in which considerable citrate synthesis takes place.

Table 4. *Citrogenase activities of cell extracts of acetic acid bacteria*

Reaction mixtures (3 ml. at pH 7·4) contained extracts of *Acetobacter* spp. and *Escherichia coli* ATCC 4157 (1 ml. of each in 0·1 M-phosphate buffer, pH 7·4); and 1 ml. of a solution (pH 7·4) containing lithium acetyl phosphate (50 μmole), oxaloacetic acid (100 μmole) and L-cysteine (50 μmole). Citrate produced was determined colorimetrically after 90 min. incubation at 28°: the values are corrected for the appropriate blank determinations on reaction mixtures from which the *Acetobacter* extract was omitted and they are the mean values of three determinations.

	Protein added in extract (μg.)	Citrate produced	
		μmole	μmole × 10^5/mg. protein/min.
Lactaphiles:			
A. acidum-mucosum	712	6·99	10·9
A. mobile	733	2·04	3·1
A. rancens	709	6·37	10·0
A. ascendens	709	9·61	15·2
A. oxydans	652	13·30	22·6
Glycophiles:			
A. capsulatum	864	*	
A. gluconicum	923	*	
A. turbidans	910	*	
A. viscosum	923	*	

* Colorimetric readings were all about 15 % lower than those of the corresponding blanks: see text.

DISCUSSION

The results presented indicate that the two groups already recognized within the genus *Acetobacter* mainly on nutritional grounds (Rainbow & Mitson, 1953; Brown & Rainbow, 1956) are also metabolically distinct. Evidence from other sources is accumulating to show that the genus *Acetobacter*, as at present constituted, contains two groups meriting generic distinction. Thus, on morphological and biochemical criteria, Leifson (1954) proposed the recognition of the genera *Acetobacter* and *Acetomonas*. This proposal is supported by the work of Shimwell (1958, 1959) and of Asai & Shoda (1958), although the Japanese workers prefer the name *Gluconobacter* to *Acetomonas*. Previous papers from this laboratory (Rainbow & Mitson, 1953; Brown & Rainbow, 1956) drew attention to the close correspondence, respectively, between our lactaphiles and glycophiles on the one hand, and the proposed genera *Acetobacter* and *Acetomonas* (or *Gluconobacter*) on the other. In so far as results obtained with relatively few strains will permit generalizations to be drawn, the present findings support the proposed new subdivision of the acetic acid bacteria into two genera with distinct metabolic patterns.

As a group, the glycophiles appear to be enzymically less well equipped than the lactaphiles, which are less exacting in their growth requirements and in their abilities to transaminate and to oxidize organic acids. This oxidative ability of lactaphilic strains may reflect a functional TCA cycle. The detection of citrogenase in their cell extracts is consistent with this hypothesis. The general inability of

glycophilic organisms to oxidize intermediates of the TCA cycle and the absence from them of appreciable citrogenase activity is an indication that in this group the TCA cycle is unlikely to function. It is noteworthy that a strain of *Acetobacter suboxydans*, the description of which shows it to be a glycophile by our criteria, is reported to lack the TCA cycle (King & Cheldelin, 1952).

Possibly the oxidative and transaminative abilities of lactaphiles are related metabolic phenomena. If the TCA cycle operates in them, the synthesis of the key amino acids, glutamate and aspartate, could take place by transamination from the corresponding keto acids generated by the cycle. This possibility is denied to the glycophiles, which fail to grow on glucose + salts + ammonia medium, but do grow when glutamate is substituted for ammonia, or when ammonia and 2-oxoglutarate are supplied together (Brown & Rainbow, 1956). The key deficiency of glycophilic organisms thus seems to be inability to synthesize 2-oxoglutarate.

Whereas lactaphilic organisms differ from glycophilic organisms in possessing greater ability to transaminate reversibly between glutamate and aspartate, both types of organism lack the ability to form α-alanine from glutamate or aspartate by transamination. However, by virtue of their aspartate β-decarboxylase system, lactaphiles can synthesize α-alanine from glutamate in two stages involving transamination to aspartate followed by enzymic β-decarboxylation. The latter process seems not to have been reported in aerobic bacteria, although the enzyme is present in *Clostridium welchii* (Meister, Sober & Tice, 1951). Our findings provide little indication of the mechanisms by which glycophilic organisms synthesize aspartate and α-alanine.

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Hydroxymethylcytosine-containing and Tryptophan-dependent Bacteriophages Isolated from City Effluents

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SUMMARY

Bacteriophages were isolated from the effluents of the cities of Oxford and Salisbury. They differ in their host range but are alike in that they contain hydroxymethylcytosine and show dependence on tryptophan for adsorption. These properties, which had been described previously only in the T-group of coliphages, are now shown to be widespread.

INTRODUCTION

The much-studied bacteriophages known as the coli T-group can be clearly divided into subgroups on the basis of morphological and biochemical differences. The members of one of these, the T-even subgroup, are indistinguishable in the electron microscope and possess the unusual pyrimidine hydroxymethylcytosine (HMC). The subgroup thus defined is also characterized by different degrees of dependence on tryptophan in adsorption. This dependence appears to be wholly absent only from strain T2.

Fildes & Kay (1957) isolated a phage from Oxford sewage which they named 'phage 3'. It was active on certain strains of *Salmonella typhi* but was later found to lyse a wide range of *Escherichia coli* strains and to have a requirement for tryptophan for adsorption to the host cells. More strains of tryptophan-dependent phages were required for comparative studies and further samples of sewage were examined. This paper describes the range of phages obtained and indicates that the phenomenon of tryptophan-dependence is widespread amongst the bacteriophage population of city effluents. It is shown to be associated with the presence of HMC.

METHODS

Sewage samples. Six (100 ml.) samples of raw inflowing sewage were taken at daily intervals at the sewage works at Oxford and Salisbury. We are greatly indebted to the officials of these works for their assistance. The samples were kept at 4° until delivered to the laboratory. Thymol was added to the Salisbury samples for transmission by post.

Isolation of phages. The sewage samples were centrifuged to remove debris and treated with thymol overnight at room temperature to diminish the bacterial population. Cultures of *Escherichia coli* 518 (Fildes & Kay, 1959) in 200 ml. of a medium containing Difco Nutrient Broth (dehydrated), 20 g.; NaCl, 7·5 g.; DL-tryptophan

(10^{-2}M), 10 ml.; Na Citrate (M), 20 ml.; Na, K phosphate buffer (0·20 M-phosphate, pH 7·6), 200 ml. made up to 1 l. with distilled water were set up and infected with 10 ml. purified sewage. After incubation at 37° overnight the lysates were centrifuged and dilutions plated on lawns of *E. coli* 518 on nutrient agar plates containing Difco Bacto-Agar, 15 g.; Difco Nutrient Broth, 20 g.; NaCl, 7·5 g.; Na citrate (M), 10 ml., per litre of water. Plaques of large and small diameters were seen, but only small ones were selected for further propagation in the phosphate + citrate + tryptophan medium. Small plaques were again selected but this time were propagated in cultures of *E. coli* 518 growing on a glucose-ammonia medium augmented with tryptophan (10^{-4}M; Kay & Fildes, 1950). Lysates of these cultures were used to prepare high-titre stocks by the procedure described by Kay (1959).

Host ranges. The twelve specimens of phage were numbered Ox-1 to 6 and Sal-1 to 6. Their host ranges and those of coliphages T 2, T 4 and T 6 were determined by placing drops of serial 10-fold dilutions on nutrient agar plates surface-seeded with bacteria. A large number of strains of *Escherichia coli* were tested and the results recorded as: full titre, equal to that given on *E. coli* 518; low, some fraction of that given on *E. coli* 518; resistant, no plaques seen at the highest concentration tested. From these observations it was clear that all the twelve phages differed from one another and from coliphages T 2, T 4 and T 6 in one or more details of their host specificities.

Morphology. The phages were prepared for examination in the electron microscope by the conventional shadowcasting procedure and by the negative contrast method of Brenner *et al.* (1959). Measurements on phages Ox-1 and Ox-6 (then named 11 F and 66 F) were given by Bradley & Kay (1960). The other phages have not been so accurately measured but have been judged solely on their overall appearance.

Hydroxymethylcytosine. Preparations of phage containing about 5×10^{12} particles in 5 ml. were centrifuged at 15,000 *g.*, the supernatant fluids discarded and the phage pellet taken up in 98 % (w/v) formic acid. After hydrolysis at 175° in sealed glass tubes the free bases were separated by paper chromatography on Whatman No. 1 paper in the *iso*propanol + HCl solvent of Wyatt (1951). Cytosine and HMC are indistinguishable in this solvent but are well separated from the other bases. The areas containing these materials were cut out, eluted in water and again chromatographed in *iso*propanol + ammonia (Hershey, Dixon & Chase, 1953), a solvent which separates cytosine from HMC. Under ultraviolet radiation the identity of the bases could be decided unequivocally.

Polyamines. The presence of the polyamines putrescine and spermidine in coliphages T 2 and T 4 was first described by Ames, Dubin & Rosenthal (1958). For the purposes of the present paper the polyamines were extracted from about 1×10^{12} phage particles into trichloroacetic acid (3 %, w/v) by heating at 45° for 1 hr. After centrifugation the supernatant fluid was evaporated to dryness and applied in a little water to paper chromatograms which were developed in a solvent consisting of the top layer from a mixture of *n*-butanol (225 ml.) + acetic acid (56 ml.) + water (225 ml.). The positions of the spots were revealed by spraying with ninhydrin solution and identified by comparison with authentic specimens of the two polyamines and with material derived from preparations of coliphage T 2.

Tryptophan dependence. A phage that shows a requirement of tryptophan for adsorption to its host bacterium depends on it likewise when presented with certain

mineral substances to which it can adsorb (Fildes & Kay, 1959). The adsorption results described in the present paper were obtained by the use of kaolin suspensions (Fildes & Kay, 1959) or by the use of bacterial suspensions treated in the same way.

RESULTS AND DISCUSSION

The properties of the twelve sewage phages are given in Table 1. The relevant details of phage 3, which was also obtained from Oxford sewage at an earlier date and coliphages T 2 and T 4 are listed for comparison. The similarities between these phages greatly outweigh the differences. Hydroxymethylcytosine, putrescine and spermidine were found in all the specimens examined. The dimensions of the

Table 1. *The properties of sewage bacteriophages*

Phage	Hydroxymethyl-cytosine	Putrescine and spermidine	Dimensions (Å)	Tryptophan requirement
Ox-1	Yes	Not tested	Head, 900 × 650 (a) tail, 850 periodicity, 30	Yes
Ox-2	Yes	Not tested	Similar to Ox-1	Yes
Ox-3	Yes	Yes	Similar to Ox-1	Slight
Ox-4	Not tested	Not tested	Similar to Ox-1	Yes
Ox-5	Yes	Yes	Similar to Ox-1	Slight
Ox-6	Yes	Yes	Head, 900 × 700 (a) tail, 850 periodicity, 30	Yes
Phage 3 (b)	Yes	Yes	Head, 900 × 650 (a) tail, 800 periodicity, 30	Yes
Sal-1	Yes	Yes	Similar to Ox-1	No
Sal-2	Yes	Yes	Similar to Ox-1	Yes
Sal-3	Yes	Yes	Similar to Ox-1	Yes
Sal-4	Yes	Yes	Similar to Ox-1	Slight
Sal-5	Yes	Yes	Similar to Ox-1	Slight
Sal-6	Yes	Yes	Similar to Ox-1	Yes
Coli T 2	Yes (d)	Yes (e)	Head, 1000–650 (c) tail, 1000 periodicity, 30	No
Coli T 4	Yes (d)	Yes (e)	Similar to T 2	Yes (f)

(a) Bradley & Kay (1960); (b) Fildes & Kay (1957); (c) Brenner *et al.* (1959); (d) Wyatt & Cohen (1953); (e) Ames, Dubin & Rosenthal (1958); (f) Anderson (1945).

particles differ by slight amounts which might be due to damage suffered during specimen preparation for electron microscopy. The over-all appearance of the virus particles is such that it would be impossible to distinguish between them in the electron microscope. All the sewage phages as isolated, with the exception of Sal-1, showed a definite requirement for tryptophan. Coliphage T 2, although of the same morphological and biochemical family, has never been described as showing tryptophan dependence.

The phages isolated during this work are probably highly unrepresentative of the types occurring in the city effluents because the procedure used was a selective one. The phosphate and citrate in the medium would ensure that all the divalent ion-requiring phages would be eliminated. On the other hand, the use of nutrient broth would make certain the retention of those phages that depend on tryptophan for

adsorption to their host bacteria. Consequently the phage population would be enriched in these types and their isolation thereby facilitated. A further selection was made when the first plaques were picked for propagation. Only small plaques were taken from a population of large and small ones. In general, large plaques are given by small phage particles and *vice versa*, the difference being due to their rates of diffusion. The selection procedure therefore favoured the isolation of large particle phages and, as was already known from work on the T-group of coliphages, only the largest of the morphological types, the T-even group, show any tryptophan effect. The present work bears this observation out and amplifies it.

Tryptophan-dependence and the presence of hydroxymethylcytosine as a nucleic acid component are major distinguishing properties of the group of phages under discussion. These properties are not found in any other group that has been examined. The size and shape of the phage particles is both characteristic and complex. They probably represent the most advanced evolutionary form of phage particle. The observation that the same type of phage exists in the effluent from Salisbury as in that from Oxford, when the two cities have quite separate water supplies and effluent systems, suggest that the type of phage in question may be very wide-spread.

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The Thermal Lability of Tryptophan-dependent Bacteriophages

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SUMMARY

Bacteriophages which are tryptophan-dependent for adsorption are also sometimes heat-labile in the presence of added tryptophan. The inactivation is due to the discharge of the phage deoxyribonucleic acid (DNA). This phenomenon is most marked at two different monovalent ion concentrations and is thought to be due to production of lesions at two different sites on the phage tail.

INTRODUCTION

Cheng (1956) reported that the 'tryptophan-dependent' phage T 4,38 was inactivated by heating to 53·5° for 1 hr., but only in the presence of tryptophan. Fildes & Kay (1959), using a similar phage, obtained the same result on heating to 45°. Electron micrographs showed disintegration of the tail tip. These phages belong to a well-defined group in which the adsorption factor requirement is not constant since tryptophan-independent mutants (tryp⁻) are easily prepared and indeed tryptophan-requiring mutants (tryp⁺) of at least one, T2, are unknown. The group is defined by containing hydroxymethylcytosine (HMC) in its deoxyribonucleic acid (DNA) and is morphologically homogeneous (Bradley & Kay, 1960). The present paper explores the heat lability of these phages further.

MATERIALS

The HMC phages known to us are the three T-even phages and others isolated from sewage (Kay & Fildes, 1961) together with several mutants which have appeared during the present work. The direct effect of tryptophan can, of course, only be demonstrated with tryp⁺ specimens which require its addition. It may be stated at once that the heat-labile phage referred to by Fildes & Kay (1959) has proved to be the only frankly labile phage in our collection. This character is however reproduced in certain mutants.

This heat-labile phage is referred to in this paper as 'K 18/3' and its derivation is as follows:

A phage was isolated by us in 1954. This was described by Fildes & Kay (1957). During 1955 and 1956 four serial propagations were made through various strains of *Salmonella typhi* under different conditions. These lysates were and are heat stable. During 1957 and 1958 a further passage was made under similar conditions and four further serial passages through a sensitive *Escherichia coli* in a glucose-ammonia

medium. The final passage K18/3/59 showed abnormalities which led to recognition of the fact that it was heat-labile. No selective pressure was applied to favour heat-labile progeny and thus it is improbable that K18/3 is a rare mutant of the original phage. Rather it would seem that it is a variant produced suddenly or gradually by changing conditions of propagation. We have been unable to reproduce this change in controlled experiments.

METHODS

The general procedure is indicated in the protocols. It follows that of Fildes & Kay (1957), but, to remove a disturbing factor, phosphate buffer was replaced by 0·011 M-tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) at pH 7·6. All solutions were made up in this buffer, which contained, as before, human albumin. DL-tryptophan was used throughout.

RESULTS

The effect of monovalent cations in the heat/tryptophan inactivation

The labile phage under discussion is referred to as 'K18/3'. This phage when suspended in 0·011 M-tris buffer and tryptophan could be heated to 45° for 1 hr. without inactivation. It was found that inactivation required also the presence of monovalent cations. An experiment was arranged as follows. Decreasing volumes of 0·25 M-NaCl in 0·011 M-tris buffer were balanced with increasing volumes of 0·011 M-tris buffer to give a series of NaCl concentrations ranging finally from 0·113 M to 0·0124 M. These were made in 1 ounce screw capped bottles and DL-tryptophan was added to 10^{-4} M, leaving a control series without tryptophan and 1 control bottle containing no NaCl. All received phage K18/3 to make $2·5 \times 10^3$ phage particles/ml. final. The bottles were immersed in water at 40°. After 30 and 60 min. the phage content was assayed by the drop method. The measure of the input phages was taken as the average of the bottles at time 0. This figure was found to be 333 phages in 4 drops (0·1 ml.). The counts of the 8 bottles containing no tryptophan ranged from 342 to 296; that of the bottle containing tryptophan but no NaCl, 321; while the results of the tryptophan-containing bottles are shown (Fig. 1) as percentage inactivated at various NaCl molarities. This experiment showed that phage K18/3 at 40° in the presence of tryptophan and NaCl was heat labile, but stable for 60 min. in the absence of either tryptophan or NaCl.

Figure 1 shows that there was a peak effect at 0·02 M-NaCl and a depression at 0·05 M, followed by a rise up to 0·12 M-NaCl. The shape of this curve of NaCl titrations is constant. Figure 1 however does not show clearly the effect of concentrations of NaCl less than that at the peak. This is indicated in Fig. 2. The percentage inactivation in 60 min. at 40° has a linear relationship to the concentration of NaCl.

In these experiments the reacting substances were confined to phage, tryptophan and NaCl at a constant pH and temperature. There was no evidence that variation in the concentration of phage or of DL-tryptophan above 2×10^{-5} M had any bearing on the result. Thus the variations in percentage inactivation must be due to changes in the concentration of monovalent cations.

The action of other monovalent cations in the presence of tryptophan was also tested. Tris buffer at 0·011 M had, as already stated, no measurable action in 60 min.

at 40° and at 0·02M the effect was very slight. However, potassium, rubidium and lithium were all active. Figure 3 shows that the action of lithium resembled that of sodium (Fig. 1).

The effect of divalent cations on the heat/tryptophan inactivation of phage K18/3

While the monovalent cations at certain precise concentrations are essential for a high rate of inactivation of this phage, the divalent cations are inhibitory by reason, apparently, of some relationship between the monovalent/divalent ion concentrations. The data already shown in Fig. 2 were used to show the percentage inactivation in 1 hr. at 40° due to different concentrations of NaCl. In two further experiments the percentage inactivation was determined in the presence of various concentrations of
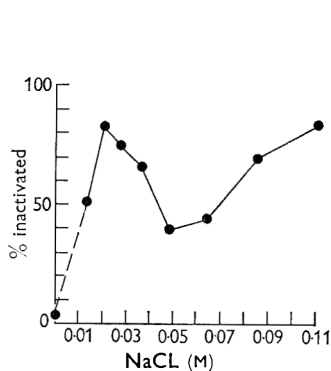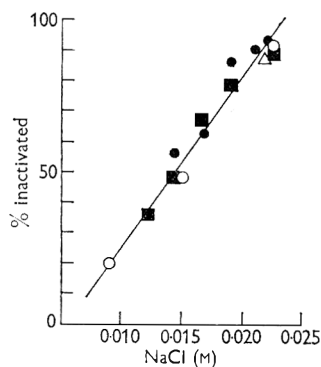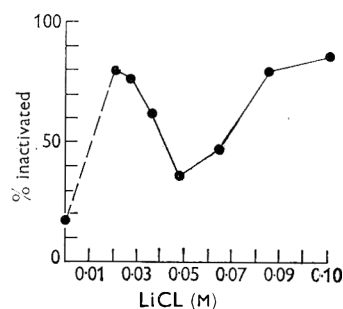
Fig. 1. Effect of NaCl on inactivation of phage K18/3 for 60 min. at 40°.

Fig. 2. Inactivation of phage Y in 60 min. at 40°. Fifteen points from four experiments. Phage Y was derived from phage K18/3 and is equally heat sensitive.

Fig. 3. Effect of lithium on inactivation of phage K18/3 for 60 min. at 40°.

magnesium ions and a constant concentration of NaCl approximating to the peak, 0·022M (Fig. 2). These results are shown in Fig. 4. It was now argued that if a certain concentration of $MgSO_4$ + an optimum concentration of NaCl produced the same degree of inactivation as a certain concentration of NaCl alone, it could be said that, for instance, 0·0222M-NaCl in the presence of 0·0001M-$MgSO_4$ left a surplus of NaCl capable of producing 61 % inactivation. From Fig. 2, this degree of inactivation was given by 0·0165M-NaCl and so it could be calculated that 0·0001M-$MgSO_4$ was 'equivalent' to 0·0222–0·0165 = 0·0057M-NaCl. Molarities of $MgSO_4$ plotted against their 'equivalents' of NaCl obtained in this way are shown in Fig. 5. It will be seen that the ratios Mg/Na vary from 1:74 to 1:34, but taking into account the technical difficulties it seems likely that the 'equivalence' ratios of the two cations are really constant.

The temperature range of the tryptophan/heat inactivation of phage K18/3

These experiments were carried out as follows. Two screw cap bottles were set up, both containing NaCl 0·025M (final) and DL-tryptophan 10^{-4}M (final). Volumes being equalized with 0·011M-tris buffer, phage K18/3 was added to $2·5 \times 10^3$ particles/ml.

final. The bottles were then immersed in water at 24°. Samples were assayed for phage at 0, 10, 20, 30, 40 and 60 min. The average of the counts of all the readings without tryptophan was taken to be the input; this was 290 plaques/0·1 ml. The individual counts of all plates were then plotted as log % input surviving at times stated (Fig. 6). It is seen that in this case 32 % of the input phage had been destroyed in 60 min. with tryptophan, but none without. Table 1 summarizes the results at various temperatures with tryptophan; no inactivation occurred without tryptophan. At 50° inactivation with tryptophan present was 99 %, but 80 % without tryptophan. Between 45 and 50° therefore a specific effect of tryptophan on heat inactivation was not demonstrable.
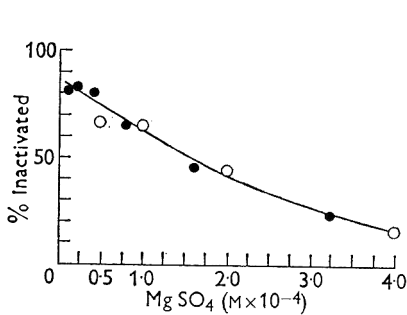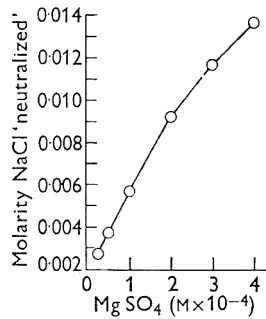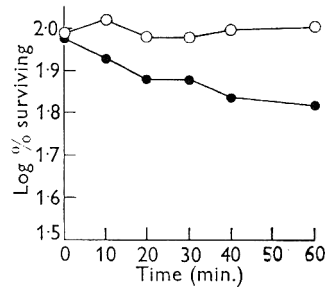
Fig. 4. Effect of magnesium on inactivation of phage Y in presence of 0·022 M-NaCl for 60 min. at 40°. Combined results of two experiments.

Fig. 5. 'Equivalence' between Mg and Na affecting the inactivation of phage Y.

Fig. 6. Rate of inactivation of phage K18/3 at 24° without tryptophan (○), with tryptophan (●).

Table 1. *Effect of different temperatures on the degree of inactivation in 1 hr.*

Temperature	Inactivation (%)
19	21
24	32
30	40
36	65
37	64
42	86
45	87

Discharge of DNA in tryptophan/heat inactivation of phage K18/3

As shown by Cheng (1956) the phage DNA is discharged during inactivation. Its release can be observed conveniently by the concomitant increase in viscosity of the solution. Four mixtures were made in screw-capped bottles all containing tryptophan. Bottle A contained finally 0·022 M-NaCl, Bottle B 0·054 M, Bottle C 0·155 M and D no NaCl. It will be noticed that the three concentrations corresponded with the first peak, depression and second rise of Fig. 1. Phage K18/3 was added to give a count of $1·1 \times 10^{11}$ particles/ml. Counts were made of the input phage at time 0 and of the survivors after 60 min. at 40°. Specimens were placed in Ostwald-type viscometer tubes at 40° at time 0 and flow-times were repeatedly measured.

The counts after the experiment showed that in Bottle A 95 % of the input had been inactivated; in Bottle B 40 %; in Bottle C 99 %; in Bottle D there was no inactivation. Figure 7 shows the rate of discharge of DNA and its dependence on the same conditions as inactivation, namely the presence of suitable concentrations of
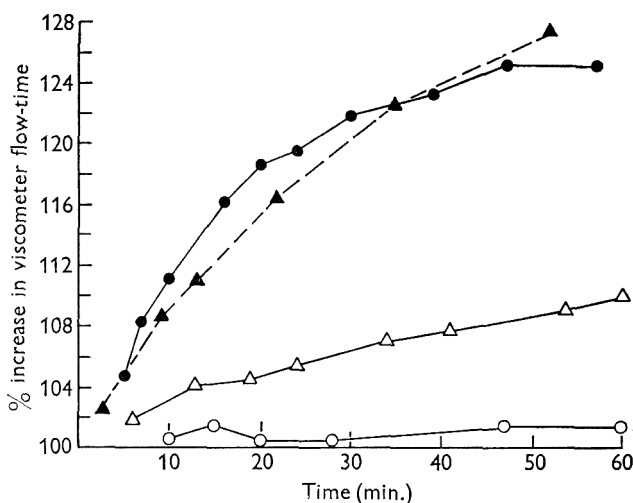

Fig. 7. Release of DNA from phage K 18/3 tryptophan mixtures at different concentrations of NaCl. Increase in flow-time in viscometer tube at 40°. No NaCl, (O); 0·022 M-NaCl, (▲); 0·054 M-NaCl, (△); 0·115 M-NaCl, (●).
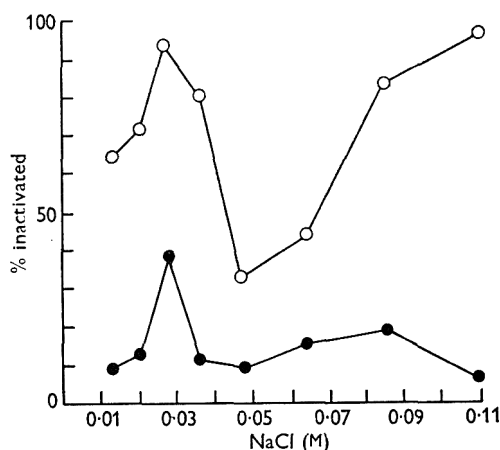
Fig. 8. Effect of varying NaCl concentrations on the inactivation of phage Y (O) and phage ZH (●) in 60 min. at 40°.

NaCl. The inactivation of phage by heat/tryptophan is known to be associated with anatomical changes in the phage tail which no doubt are the immediate causes of the discharge of DNA. It seems however probable that the anatomical changes are not the same at all NaCl concentrations and that the depression in the middle of Fig. 1 is due to a change in the quality of the lesion.

The behaviour of certain mutants of phage K 18/3

Phage K 18/3 is a direct descendant of the original 'Phage 3' isolated by us in 1954. The original phage and a recent repropagation under current conditions were heat stable. Four serial propagations of this phage for stock purposes remained heat stable but after five further propagations it was noticed that the heat-labile strain (K 18/3) had appeared. It was found that isolated plaques behaved differently with isolated colonies of the coli cultures used. In this way two different phages were separated on host-range differences. One of these, 'Y', was sensitive to 37°; the other, 'ZH', was much more stable.
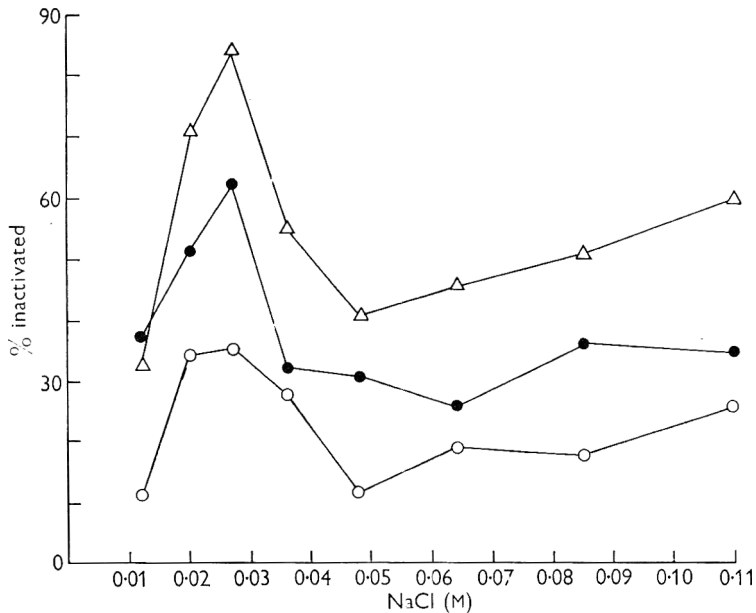

Fig. 9. Effect on inactivation of phage ZH of prolonged time at 40°.
1 hr. (○), 2½ hr. (●), 5 hr. (△).

Phage K 18/3 was heated in the presence of tryptophan and 0·025 M-NaCl for 12 hr. at 45°. At the beginning there were 1 3 × 10¹⁰ phages/ml.; at the end 3·1 × 10⁷. This remnant propagated in the usual way produced a lysate from which plaque selection was made and phage F.H45.T produced. This phage proved to be stable at 37° for 40 min.

The two phages thus separated from K 18/3 were more heat stable than the rest of the population. When phage ZH was tested for stability at various NaCl molarities it was found (Fig. 8) to show a small but sharp rise in lability at the same peak (0·027 M) as its fellow Y; but the secondary rise in the range 0·11 M did not appear or at least was insignificant. When also the action of heat on phage ZH was more prolonged, i.e. to 5 hr. (Fig. 9), no less than 84 % was inactivated at 0·027 M-NaCl, but still the secondary rise was much less than in the heat-labile variety (cf. Figs. 1, 3, 8). It thus appears that the more heat-stable phages may differ from the more labile in two ways: firstly, in being slower to respond to the heat inactivation at 0·027 M-NaCl and, secondly, in being less susceptible to the inactivation at 0·11 M-NaCl.

DISCUSSION

The property of marked heat-lability in the presence of tryptophan and monovalent ions does not seem to be widespread among the HMC phages. In our collection only one out of twelve phages isolated from sewage was sensitive (excluding its mutants) and Cheng (1956) mentioned only one phage of this type. Our phage K 18/3 only shows its lability when the ionic environment is precisely adjusted and when tryptophan is present. Otherwise it is as stable as its parent strain. In any case the stability to heat is a relative matter. Comparison between our stable and unstable variants isolated from K 18/3 shows that the difference is one of rate of inactivation rather than of complete resistance or complete sensitivity. A more subtle difference between the two phages is revealed when their inactivation is studied with respect to the monovalent ion concentration. It is then seen that even when the degree of inactivation at the low NaCl concentration is increased by prolonging the time of the experiment the inactivation at the high NaCl concentration does not increase to that of the labile phage variant. We therefore conclude that though the actions of NaCl at the high and low concentrations are similar, namely to produce a lesion in the presence of tryptophan which results in discharge of DNA from the phage, nevertheless the actions at the two NaCl concentrations are exerted at different sites in the phage tail.

Our findings on the effect of monovalent and divalent salts on the action of heat and tryptophan on HMC phages are, broadly, similar to those of salts and heat on phages which are indifferent to tryptophan (cf. Gard & Maaløe, 1959). The lesions produced by heat in a certain ionic environment may well be the same with many phages. But in the HMC phages tryptophan is a necessary activator of the action.

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The S-R Variation in *Lactobacillus casei*

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SUMMARY

Colonies of four strains of *Lactobacillus casei* var. *casei* and three strains of *L. casei* var. *rhamnosus* investigated may develop rough outgrowths while growing on a medium containing no fermentable carbohydrate. Colonies do not form visible outgrowths when growing on the same medium containing more than 0·5 % (w/v) glucose. Rough elements may be present in the latter colonies, but because of lack of a selective advantage do not show phenotypically. The rough outgrowths possess the characteristics of R variants. The biochemical reactions of the R variants are identical to those of the parent S form. The mutational origin of the R variants from wild S types is demonstrated by a method involving the direct observation of rough clones. The mutation rate involved is presented for six of the strains. In continuous culture in broth both the S and the R types are stable. On agar the R variant is also stable.

INTRODUCTION

In a study of carbohydrate utilization by *Lactobacillus casei*, strains were plated on solid media containing sugars which they normally do not ferment. Colonies which arose were studied daily for the presence of papillae which might indicate the presence of clones capable of utilizing the extra source of energy provided by the carbohydrate. No papillae of this nature were detected, but we were struck by the number of old colonies which had rough outgrowths projecting from their margins. Many workers (see Barber & Frazier, 1945; Davis, 1956) have encountered colonial variants of lactobacilli (see Hadley, Bunting & Delves, 1930, for illustrations), but no quantitative experiments have been reported to establish how variants derive from one another in a particular environment. Most of the variants described have belonged to the subgenus *Thermobacterium* (Rogosa & Sharpe, 1959). If strains of the *Bacillus acidophilus* group I of Hadley *et al.* (1930) are accepted as *L. casei*, then these workers must be regarded as the first to have succeeded in systematic attempts at isolating morphological variants of this species. Barber & Frazier (1945) and McDonald & Frazier (1951) also attempted the isolation of colonial variants of *L. casei*. The former failed with their type strain 28 but succeeded with eight other unnamed strains; McDonald & Frazier were unsuccessful. Weinstein, Anderson & Rettger (1933) attempted the isolation of rough variants from thirty-seven unidentified oral strains of lactobacilli, they were successful with only one of the strains. It was decided to investigate the nature of the rough outgrowths present on colonies of *L. casei*.

METHODS

Organisms. Strains C2, C9 and H2 were kindly supplied by Dr M. Elisabeth Sharpe (National Institute for Research in Dairying, Reading, Berkshire, England). The first two represent the serological groups B and C, respectively, of *Lactobacillus casei* var. *casei* (Sharpe & Wheater, 1957; Rogosa & Sharpe, 1959) and the last is a *L. casei* var. *rhamnosus* strain (Rogosa *et al.* 1953). A further two strains of *L. casei* var. *casei* (no. 300 and 316) and two strains of *L. casei* var. *rhamnosus* (no. 542 and 430), isolated locally from human saliva, were investigated. The latter strains were subjected to repeated single colony isolation to ensure purity. Stock cultures were stored in the lyophilized state, but during the period of these experiments they were maintained in a litmus milk medium (Davis, 1935) at 4° and subcultured at three-monthly intervals. Bacteriophages 300 and 316 were isolated from sewage (Coetzee, de Klerk & Sacks, 1960) and are lytic for *L. casei* strains 300 and 316, respectively. The phage techniques used were those of Adams (1956).

Media. The plating media consisted of a tomato glucose agar and the same medium without the tomato juice and glucose called medium I. The tomato glucose agar had the following composition: agar (Difco), 20 g.; peptone (Difco), 15 g.; Yeastrel (Brewers' Food Supply Company Ltd, Edinburgh), 3 g.; Tween 80, 0·1 g.; salt solution (Rogosa & Mitchell, 1950), 5 ml.; sodium acetate, 5 g.; potassium di-hydrogen orthophosphate, 5 g.; di-ammonium hydrogen-citrate, 2 g.; glucose, 20 g.; tomato juice, 100 ml.; distilled water to 1000 ml. The medium was adjusted to pH 6·1 and sterilized by autoclaving at 121° for 20 min. The tomato juice was prepared according to the method of Briggs (1953). The concentration of reducing sugars in the tomato juice was determined by the method of King, Haslewood, Delroy & Beale (1942), and the constituent sugars were identified by chromatography (Horrocks & Manning, 1949). The broth used was that of medium I. As tomato juice and Yeastrel are antigenic (Sharpe, 1955*a*) the medium used for growing organisms to give antigens for injection into rabbits had the following composition: peptone (Difco), 10 g.; Tween 80, 0·1 g.; glucose, 20 g.; malt extract (Difco), 1 g.; distilled water to 1000 ml.; adjusted to pH 6·1 and sterilized by auto-claving at 121° for 20 min.

The media and methods used for identifying strains of lactobacilli were those described by Wheater (1955). Cultures were incubated at 37° in an atmosphere of 100 % CO_2.

Serology. Antigens for rabbit inoculation and the corresponding antisera were prepared according to the methods of Sharpe (1955*b*). Agglutination and agglutinin-absorption tests were done according to the methods of Mackie & McCartney (1953). Where spontaneous clumping occurred, the organisms were suspended in distilled water and the serum dilutions made in 0·2 % (w/v) NaCl solution.

Continuous culture. Continuous cultures of organisms were started by subculturing 0·05 ml. of a 10^{-7} dilution of an overnight growth in broth into 1000 ml. prewarmed broth. This inoculum when plated on medium I yielded about 3 colonies for both smooth and rough growths. At the first indication of growth (usually after 36 hr. at 37°) cultures were well-shaken to homogenize them and 0·2 ml. subcultured into a further 1000 ml. of prewarmed broth. By repeating this process at 24 hr. intervals, cultures never reached their maximum concentrations and could be maintained in

exponential growth. Mixtures of smooth and rough organisms of strain C 9 were also maintained in logarithmic growth by the same method.

Mutational origin and mutation rates of organisms in rough outgrowths. For reasons to be mentioned the enumeration of individual organisms was avoided as far as possible and the work on mutation and mutation rates was based on the modification by Ryan, Schwartz & Fried (1955) of the method of Luria & Delbrück (1943) for determining mutation rates of bacteria. This method is independent of the number of mutants and also of the growth rates of the parent and mutant. It is based on the assumption that the clonal progeny of each mutation with a selective advantage, occurring during the development of a colony, will become visible as a papilla. The mean number of mutations/colony may then be determined by direct observation. On the assumption that mutations are randomly distributed, the mean number of mutations/colony can also be calculated from the proportion of colonies without papillae. Rough outgrowths may be regarded as papillae of a different nature which possess certain selective advantages. This reasoning has been applied to other systems (Coetzee & Sacks, 1960) and will be used here. Cultures were accordingly seeded on medium I to yield about 50 colonies/plate. This was done by pipetting 0·1 ml. of suitable dilutions and rubbing to dryness with a sterile glass rod. The number of outgrowths/colony was recorded daily until no more appeared. Plates were incubated between the counting sessions. At this stage the total number of colonies was counted and a number of entire colonies, with no visible outgrowths, were individually streaked on medium I plates and incubated to discover any hidden rough elements within smooth colonies. These would be expected to appear as entirely rough colonies. After scanning with a dissecting microscope the proportion of plates showing no rough colonies was used in the Poisson distribution to obtain an estimate of the mean number of hidden rough growths/colony.

The total population of smooth colonies with no outgrowths at the end of the period of observation was estimated with the use of a Petroff–Hauser counting chamber and a phase-contrast microscope according to the method of Ryan *et al.* (1955). Cytological observations were made on bacteria stained with acid Giemsa according to the method of Bisset (1950).

RESULTS

On tomato glucose agar well separated 48 hr. colonies were circular, about 2 mm. in diameter, dome-shaped with an entire edge. The surface was very finely granular and glistening and the colonies white and opaque. On medium I the colonies were smaller, less raised and had a smooth glistening surface (Pl. 1, fig. 1). On further incubation colonies on tomato glucose agar became larger but did not change in character. On medium I they became slightly larger and flatter, and developed on their surfaces various smooth excrescences of unknown nature. These excrescences, on subculture on medium I, yielded colonies identical in appearance with the smooth parent and were not investigated further. In addition to these excrescences, rough outgrowths literally exploding anywhere from the surfaces or margins of colonies may become discernible in some cases after incubation for about 72 hr. When first seen they might consist of a few protruding whispy strands of growth resembling the curled hair-lock appearance associated with colonies of *Bacillus anthracis* (Pl. 1,

figs. 2, 3) or they might be much more impressive florid structures (Pl. 1, fig. 4). Whatever the beginning, this growth was out of step with the remainder of the colony and continued to increase in size when the rest of the colony had come to a standstill. For this reason it was important, in quantitative experiments, to detect outgrowths at an early stage of development before they coalesced with others on the same colony. The surfaces of these outgrowths were extremely rough and contrasted sharply with the adjoining surfaces of the colony. On a few occasions colonies with rough sectors were also seen. To determine why colonies produced outgrowths on medium I but not on tomato glucose agar, outgrowth formation by strain 300 (the strain with the highest incidence of outgrowths/colony) was studied on medium I at different concentrations of glucose (0·1 %, w/v, to 4·0 %, w/v) and on medium I with concentrations of tomato juice ranging from 10 % (v/v) to 40 % (v/v). These colonies were observed for 14 days; the results are presented in Table 1.

Table 1. *Results of an experiment to determine outgrowth formation of* Lactobacillus casei *strain* 300 *on various media*

Plates were inoculated with a dilution of *L. casei* strain 300 yielding about 50 colonies/plate. About 500 colonies on each medium were studied for 14 days. Numbers in brackets denote the concentration (w/v) of sugars present in media.

Media	No. outgrowths/colony
Medium I (0)	0·40
Medium I + 10 % (v/v) tomato juice (0·24)	0·41
Medium I + 20 % (v/v) tomato juice (0·48)	0·39
Medium I + 40 % (v/v) tomato juice (0·96)	0
Medium I + 0·1 % (w/v) glucose	0·39
Medium I + 0·25 % (w/v) glucose	0·40
Medium I + 0·5 % (w/v) glucose	0·38
Medium I + 1·0 % (w/v) glucose	0
Medium I + 2·0 % (w/v) glucose	0
Medium I + 4·0 % (w/v) glucose	0
Tomato glucose agar (2·24)	0

The total concentration of reducing sugars present in the tomato juice used was 2·4 g./100 ml. As the method of preparation of the juice involved the hydrolysis of any sucrose, this figure reflects the total concentration of sugar present. Paper chromatography proved the bulk of the latter to be glucose. Up to a concentration of 0·5 % (w/v) sugar the mean number of outgrowths/colony on medium I + glucose and on medium I + tomato juice was similar to that on medium I. Above this concentration the incidence was zero. The presence of 2·24 % (w/v) sugar in tomato glucose agar thus appeared to account for the absence of outgrowths on this medium.

When rough areas of colonies were subcultured on to medium I agar they gave rise to typical medusa-head colonies (Pl. 1, fig. 5; Bisset, 1938; Davis, Bisset & Hale, 1955). Apart from an increase in size, these medusa-head colonies underwent no other change in morphology with longer periods of incubation. They also bred true when streaked for single colonies on medium I agar; no smooth colony progeny were ever detected in numerous platings. On tomato glucose agar subculture rough outgrowths of strains C9 and 316 yield smoother colonies, closely resembling the original smooth parent colony, but slightly duller in appearance. Rough outgrowths from strain 300 persisted as rough colonies on tomato glucose agar. To prove that

differences in appearance on the two kinds of media are not due to selection of variants, a medusa-head colony of C9 was repeatedly restreaked on medium I and a single colony finally picked off and suspended in sterile distilled water. From this suspension suitable dilutions were made and from the same dilution tube 0·1 ml. volumes were seeded on plates of tomato glucose agar and medium I. After 48 hr. the colonies were examined and counted. There were 326 colonies on medium I and 311 colonies on tomato glucose agar. Those on medium I were all typical medusa-head colonies while the colonies on tomato glucose agar were all smooth dome-shaped colonies, only slightly duller in appearance than the original smooth colonies.

In broth smooth colonies produced a uniform turbidity with a slight finely granular deposit after overnight incubation. Subcultures from rough outgrowths produced a granular stringy growth at the bottom of the tube and a clear supernatant fluid. In 0·85 % (w/v) NaCl solution rough growths were unstable and soon settled out. Microscopically the organisms from rough areas, whether on agar or in broth, consisted of a tangled mass of chains comprising thousands of bacilli. The appearance was that of a long ribbon folded upon itself. Chain formation of rough growths occurred despite the presence of smooth-colony inducing agents (Tween 80, acetate) in the media used (Rogosa & Mitchell, 1950; Rogosa *et al.* 1953; Davis, 1956; Rogosa & Sharpe, 1959). The addition of tryptophan (Green, Dodd & Radike, 1955) also had no effect on chain formation. Organisms from smooth areas were usually single or in short chains of 3 or 4 bacilli.

Continuous culture experiments were done with purified rough and smooth growths of *L. casei* strains 300, C9 and 316 maintained in exponential growth for 400 hr. and plated for single colonies on 10 plates each of medium I at 24 hr. intervals. Both smooth and rough elements were perfectly stable under these conditions; no primarily smooth or medusa-head colonies appeared on the plates from rough or smooth continuous cultures, respectively. A similar atavistic deficiency was noted by Tracy (1938) for R variants of *L. plantarum*. Kopeloff (1934), on the other hand, could readily select S variants from R cultures of *L. acidophilus*, but not vice versa. In continuous cultures started with mixtures of smooth and rough growths of *L. casei* strain C9 the smooth-colony growth overgrew and entirely replaced the medusa-head colony forming growth within 144 hr. Because of chain formation of the rough growths these experiments were subject to gross errors of sampling and enumeration. Yet the above results were obtained when platings of initial inocula indicated a starting ratio of ten medusa-head colonies to one smooth colony. Under these conditions cultures were almost certainly initiated with a great excess of rough organisms.

Cultures from smooth colonies of *Lactobacillus casei* strains 300 and 316 are susceptible to lytic phages 300 and 316 respectively. Rough outgrowths of strain 316 did not adsorb phage 316, but the efficiency of plating of phage 300 on rough cultures of strain 300 was identical to that of the smooth variant. No phage was available for testing on the other strains investigated. The biochemical reactions and growth temperatures of purified rough growths were identical to those of the parent smooth strain.

Results of agglutination and agglutinin absorption tests of sera prepared against smooth and rough variants of strains C9, 300 and 316 are presented in Table 2. These results indicate antigenic differences between smooth and rough growths of all

three strains. The difference in each case appears to be an antigenic deficiency of the rough growths. This is the usual change associated with the S-R variation (*Topley & Wilson's Principles*, 1955). On no occasion, in all the platings done from stock cultures during these experiments, were primary medusa-head colonies detected. All

Table 2. *Results of agglutination and agglutinin absorption tests on antisera prepared against smooth and rough growths of* Lactobacillus casei *strains* C9, 300 *and* 316

Antigens were suspended in distilled water and serum dilutions made in 0·2 % (w/v) NaCl solution. Equal volumes of antigen and antiserum were mixed and tests were kept in a water bath at 50° for 6 hr. and then at 4° overnight. Agglutinin absorptions were done by adding an excess of tightly packed heated antigen to a dilution of serum corresponding to 64 times the concentration of its titre. The contents of these tubes were well mixed and kept at 37° for 4 hr. The controls with 0·2 % (w/v) NaCl solution without serum were satisfactory in all cases.

Antiserum	Serum absorbed with	Antigen	Reciprocal of titre
C9 smooth	—	C9 smooth	8192
C9 smooth	—	C9 rough	4096
C9 smooth	C9 rough	C9 rough	>128
C9 smooth	C9 rough	C9 smooth	512
C9 rough	—	C9 rough	2048
C9 rough	—	C9 smooth	1024
C9 rough	C9 smooth	C9 smooth	>32
C9 rough	C9 smooth	C9 rough	>32
300 smooth	—	300 smooth	8192
300 smooth	—	300 rough	4096
300 smooth	300 rough	300 rough	>128
300 smooth	300 rough	300 smooth	512
300 rough	—	300 rough	2048
300 rough	—	300 smooth	512
300 rough	300 smooth	300 smooth	32
300 rough	300 smooth	300 rough	>16
316 smooth	—	316 smooth	4096
316 smooth	—	316 rough	4096
316 smooth	316 rough	316 rough	>128
316 smooth	316 rough	316 smooth	512
316 rough	—	316 rough	2048
316 rough	—	316 smooth	128
316 rough	316 smooth	316 smooth	>4
316 rough	316 smooth	316 rough	>4

Table 3. *Total counts of organisms present in* 14-*day smooth colonies of various strains of* Lactobacillus casei *with no visible outgrowths*

Six entire colonies of each strain were cut out with the underlying agar and thoroughly emulsified in separate 1 ml. volumes of distilled water. Organisms present in samples were then counted in a Petroff–Hauser chamber by means of a phase-contrast microscope.

Strain	Mean population of 6 colonies (N)
C2	$7·0 \times 10^7$
C9	$1·8 \times 10^8$
300	$7·4 \times 10^7$
316	$1·3 \times 10^8$
H2	$4·0 \times 10^7$
430	$5·5 \times 10^7$
542	$7·0 \times 10^7$

Table 4. *Results of two sets of experiments with* Lactobacillus casei *strains C9, 300 and 316 to determine the mutational origin and mutation rates of rough from smooth growths*

	Experiment 1			Experiment 2		
	Strain C9	Strain 300	Strain 316	Strain C9	Strain 300	Strain 316
	Variant colonies (no.)			Variant colonies (no.)		
No outgrowths	233	250	549	1208	576	1786
1 outgrowth	21	100	55	129	226	138
2 outgrowths	2	24	5	10	47	9
3 outgrowths	0	1	0	0	6	0
4 outgrowths	0	0	0	0	1	0
5 outgrowths	0	0	0	0	1	0
Mean no. outgrowths/colony (m)	0·1	0·4	0·11	0·11	0·4	0·08
Probability of drawing a similar sample of outgrowths from a Poisson distribution with m as mean (P)	0·40	0·62	0·25	0·29	0·89	0·15
No. of 14-day-old colonies with no outgrowths streaked out	30	30	30	30	30	30
No. of these plates revealing rough colonies	0	2	1	0	2	1
Mean no. concealed rough growths/colony (mc)	0	0·07	0·03	0	0·07	0·03
Mutation rate/bacterium/generation (m + mc)ln2/N	3·9 × 10⁻¹⁰	3·8 × 10⁻⁸	7·5 × 10⁻¹⁰	4·3 × 10⁻¹⁰	3·8 × 10⁻⁸	5·9 × 10⁻¹⁰

N, mean total count of six 14-day colonies showing no outgrowths. Colonies on medium I agar were examined daily for rough growths. No new rough elements appeared after 14 days' incubation.

Table 5. *Results of experiments with* Lactobacillus casei *strains* C2, H2, 430 *and* 542 *to determine the mutational origin and mutation rates of rough from smooth growths*

	Variant colonies (no.)			
	Strain C2	Strain H2	Strain 430	Strain 542
No outgrowths	1360	5015	2918	1299
1 outgrowth	139	2	235	241
2 outgrowths	4	0	7	22
3 outgrowths	0	0	1	4
Mean no. outgrowths/colony (m)	0·1	—	0·08	0·19
Probability of drawing a similar sample of outgrowths from a Poisson distribution with m as mean (P)	0·25	—	0·61	0·69
No. of 14-day old colonies with no outgrowths streaked out	20	45	20	20
No. of these plates revealing rough colonies	1	0	0	1
Mean no. concealed rough growths/colony (mc)	0·05	0	0	0·05
Mutation rate/bacterium/generation (m+mc)1n2/N	$1·5 \times 10^{-9}$	—	$1·1 \times 10^{-9}$	$2·4 \times 10^{-9}$

N, mean total count of six 14-day colonies showing no outgrowths. Colonies on medium I agar were examined daily for rough growths. No new rough elements appeared after 14 days' incubation.

colonies were initially smooth; rough outgrowths were generally first detected after 4 days, but sometimes appeared as late as the 13th day of incubation.

Counts of the total bacterial population of 14-day smooth colonies on medium I with no outgrowths are presented in Table 3. These counts may be regarded as reliable because smooth colonies formed homogeneous suspensions mainly composed of single organisms. The chains present were short and constituent organisms could be counted.

Results of the enumeration of outgrowths are presented in Tables 4 and 5. Because of the great crowding on plates used to determine the number of hidden rough growths/colony, these estimates must be regarded as minimal. The *P* values given in Tables 4 and 5 represent the chances of drawing similar samples from Poisson distributions with means equal to the mean number of growths/colony. The values obtained are taken to indicate that the outgrowths were randomly distributed and support the theory of the mutational origin of the events which started the rough clones (Ryan *el al.* 1955). Mutation rates/bacterium/generation (Luria & Delbrück, 1943) are also presented in Tables 4 and 5; these are low and may be overestimated. The reason is that acid Giemsa staining of organisms in smooth growths reveal from 1 to 4 chromatinic bodies/organism and the mutation rate is expressed as such, and not per nucleus.

There is a possibility that rough variants did arise during colony formation on tomato glucose agar but failed to register phenotypically because of lack of a selective advantage. This was investigated by streaking fifty entire 5-day colonies of strain 300 from tomato glucose agar on to 4 plates each of medium I agar. After incubation for 48 hr. plates were examined with a dissecting microscope for pure medusa-head colonies. Plates representing ten colonies had these rough colonies present. Application of the zero Poisson distribution gives 0·22 for the mean number of unobserved rough clones present in 5-day smooth colonies on tomato glucose agar. Very similar results were obtained when 5-day colonies of strain 300 on medium I containing 2 % (w/v) glucose were likewise plated on medium I agar.

DISCUSSION

The rough outgrowths present on colonies of *Lactobacillus casei* form unstable suspensions in 0·85 % (w/v) NaCl solution and broth, and differ antigenically from the parent colony. They thus conform to R variants of bacteria described by Arkwright (1920, 1921). Arkwright (1924), Hadley (1926) and Burnet (1927) also demonstrated that S and R variants of a strain might have different bacteriophage specificities. Strain 316 obeys this rule in that only the S variant adsorbed phage 316. Both the S and the R forms of strain 300 are susceptible to phage 300. This indicates that the change in surface structures associated with the S-R variation is not necessarily of the same nature or extent in all strains. Arkwright (1920) noted this in his first communication on the S-R variation and Bissett (1938) could not demonstrate serological differences between S and R variants of a strain of *Shigella dysenteriae* (Flexner).

No biochemical differences were detected as between the S and R variants of strains examined. Rogers (1934) also observed that the fermentation reactions and growth temperatures of S and R variants of a strain of *Lactobacillus acidophilus* were identical. Hadley *et al.* (1930) noted biochemical differences between S and R variants of their group 1 (? *L. casei*) strains. Tracy (1938) encountered a strain of

L. plantarum in which the S-R variation was associated with a loss of saccharolytic properties and Pederson (1947) claimed that *L. acidophilus* and *L. casei* were simply rough and smooth variants of the same organism. Pederson's claim has never been confirmed and was criticized by Davis (1956) on grounds of faulty technique.

Entire populations of lactobacilli may change their colonial form abruptly when plated on different media. This has been demonstrated by Rogosa & Mitchell (1950), McDonald & Frazier (1951) and by Green *et al.* (1955). Similar changes have been produced with the colonial morphology of *Lactobacillus casei* strains C 9 R and 316 R. This alteration of morphology of a population is not the result of genetic changes but simply a difference in phenotypic expression in different environments. The rough growths encountered in all strains of *L. casei* investigated here started as randomly distributed events during the development of the colony and may be regarded as mutations which were selected by the particular ecological conditions prevailing in the colony and its environment. The results obtained indicate that the absence of fermentable carbohydrates in medium I agar provided some selective advantage for the R mutants. Hadley *el al.* (1930), however, observed smooth colonies of their group I (? *L. casei*) to undergo 'marginal dissociation' to R forms on a glucose infusion agar. Barber & Frazier (1945) selected R variants of *L. casei* by repeated subculture in carrot liver broth at 48°, with platings on the agar. Weinstein *et al.* (1933) used a tomato juice broth and agar without any additional carbohydrate. A selective advantage for mutants is the essence of the papillary method of determining mutation rates. This was shown in the present work by finding genetically rough clones concealed in colonies of *L. casei* strain 300 on tomato glucose agar and on medium I + 2 % (w/v) glucose. Apart from *L. casei* var. *casei* strain 300 which has a high S-R mutation rate and *L. casei* var. *rhamnosus* strain H 2 which forms very few outgrowths, the remaining strains of both species had fairly uniform S-R mutation rates and the latter feature did not appear to possess any taxonomic significance. The mutation rates from S to R are low for morphological variants of bacteria (Coetzee, 1959) but there is no reliable independent method of confirming these results. The reason is that all other methods of determining these mutation rates involve enumeration of R organisms. Because of chain formation viable counts are misleading. Total counts are also unsatisfactory because, despite thorough shaking of cultures of rough growths, numerous chains of organisms extending across the microscopic field are invariably present and constituent organisms cannot be counted.

No back mutation of the R to the S form was demonstrated on agar or in continuous culture. The S form is perfectly stable in continuous culture, but if R variants should derive from the S form in Nature the latter must possess some selective advantage to maintain itself as the wild or modal type (Rogosa & Sharpe, 1959). With due regard to the limitations of the experiments, the fact that in mixed continuous cultures the S form invariably entirely replaced the R form may be taken to indicate that the former possesses a shorter generation time (Armitage, 1952). Barber & Frazier (1945) maintained that S forms of *L. bulgaricus* divided more rapidly than the corresponding R variants. With the R cultures studied here the growth rates could not be determined because of the difficulties of enumeration previously mentioned. Metabolically the two forms appear similar, but it may be that in Nature the individualistic S form possesses some other subtle advantage over the gregarious long-chain R mode of existence.

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EXPLANATION OF PLATE

PLATE 1

Colonial morphology of *Lactobacillus casei* strains on medium I agar; magnification about ×25.

Fig. 1. 2-day colonies of strain 300.

Fig. 2. 8-day colony of strain 300 showing a number of smooth excrescences on its surface and one early rough outgrowth.

Fig. 3. 8-day colony of strain C9 showing a number of excrescences and at least four rough outgrowths.

Fig. 4. 10-day colony of strain 316 showing a number of excrescences and at least two rough outgrowths of different ages.

Fig. 5. 2-day medusa-head colony of strain C9. This is a subculture of a rough outgrowth from a C9 colony.

Plate 1

H. C. DE KLERK AND J. N. COETZEE
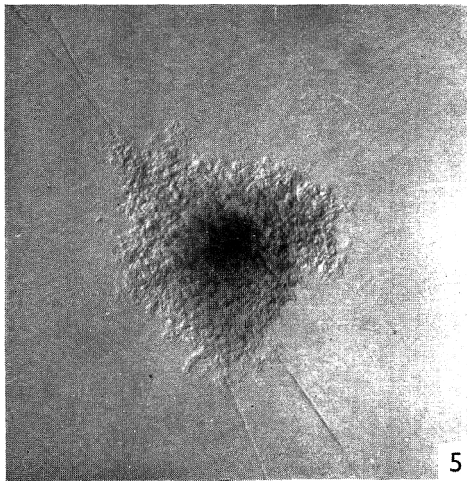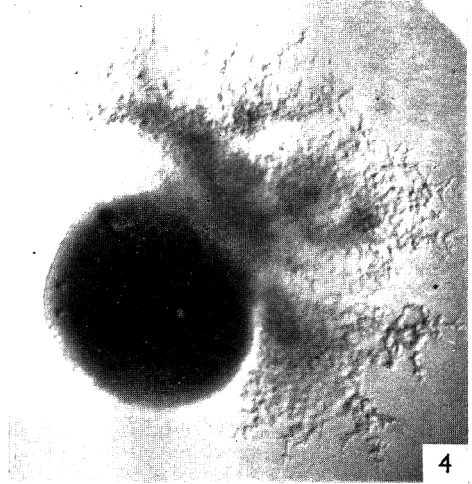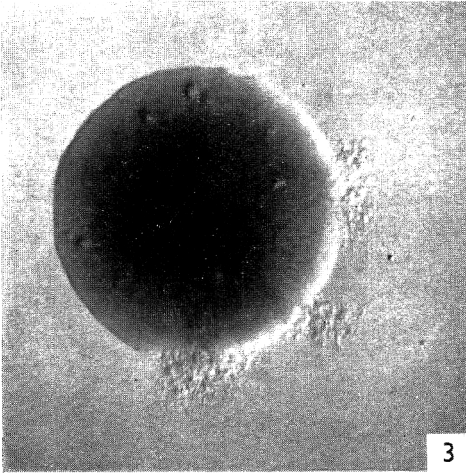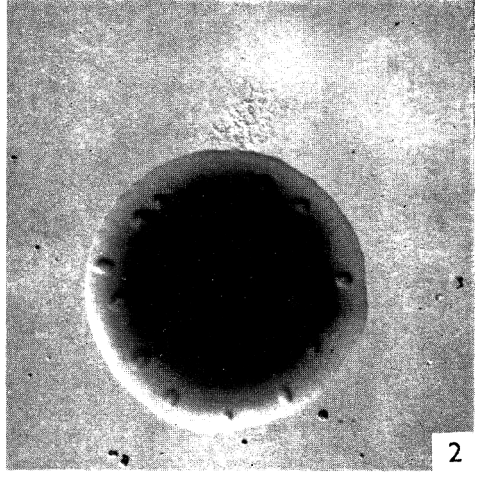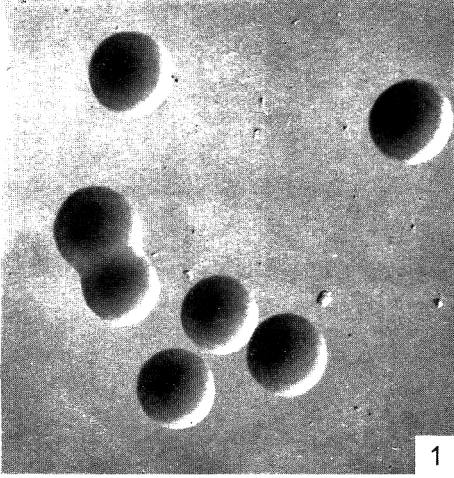
Curly Flagellar Mutants in Salmonella

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SUMMARY

A curly flagellar mutant obtained from a strain of *Salmonella typhimurium* was unstable and repeatedly dissociated 'curly' and normal subclones. Examination of flagellar antigens of the normal and the curly flagellar subclones demonstrated that the changes in flagellar shape corresponded exactly with phase variation: subclones with curly flagella were always in phase-1 (antigen-*i*), those with normal flagella were in phase-2 (antigen-*1,2*). In transduction from a normal flagellar strain to the curly phase-1 strain, transductional clones with normal flagella were isolated. The transductional clones showed the antigen of the donor in phase-1 and that of the recipient in phase-2. This indicates that the phase-1 curly determinant is closely associated with the phase-1 antigen type determinant, H_1.

Seven curly mutants were obtained from a strain of *Salmonella abortus-equi*: one with antigen-*a* (phase-1), and four with antigen-*e,n,x* (phase-2), and two with antigen-*1,2* (phase-2) from a transductional recombinant given antigen-*1, 2*. Transductional analysis with these strains showed that the phase-2 curly determinant is closely associated with the phase-2 antigen type determinant, H_2; and the phase-1 curly determinant with H_1. In cross-absorption experiments with antisera prepared against flagella of either normal or curly mutant, no antigenic difference between normal and curly flagella could be detected. It is inferred that H_1 and H_2 are the primary structural determinants of flagellar protein in phase-1 and phase-2 respectively; mutation in H_1 or H_2 may cause an altered configuration of flagellar protein, resulting in a change in antigenic type, or in flagellar shape, or it may cause the failure of flagellar morphogenesis. Attempts to obtain recombination by transduction between the curly flagellar determinants in each of the phases have been unsuccessful; this suggests that in each phase the mutant sites of the curly types are very closely linked or identical.

INTRODUCTION

The shape of bacterial flagella is conditioned by environmental and inherent factors. In cases where the effect of environment is not remarkable, their inherent characteristic shapes are regarded as useful traits for the identification of bacterial species (Leifson & Hugh, 1953; Leifson & Palen, 1955). Several mutant types have been described. For instance, mutants with flagella having a wavelength half that found in the wild-type have been discovered in some strains of Proteus (Leifson, Carhart & Fulton, 1955), Salmonella (Leifson & Hugh, 1953) and Chromobacterium (Leifson, 1956). This kind of mutant has been called 'curly'.

Recently, an investigation on flagellar regeneration (Kerridge, 1959) demon-

strated that curly flagella were produced when regeneration occurred in a medium containing p-fluorophenylalanine in place of phenylalanine, and that bacteria with such abnormal flagella were almost entirely non-motile. Bacterial flagella are composed of a fibrous protein called flagellin (Astbury, Beighton & Weibull, 1955). The regeneration experiment suggests that the incorporation of an abnormal amino acid during the synthesis of flagellin causes an alteration of flagellar curvature. If a similar biochemical mechanism is involved in the production of curly flagella of genetic origin, it might be taken as a unique character for the investigation of gene control of protein synthesis, including the coding problem. The genetic analysis of curly flagellar mutants reported in the present paper was undertaken with such expectation.

METHODS

The salmonella strains mainly used in the present work were *Salmonella typhimurium* strain SW 577 and *S. abortus-equi* strains SL 23 and SJ 25. SW 577 is the strain reported by Leifson & Hugh (1953) as a curly flagellar strain. Its flagellar morphology and clonal instability were first described by them. SL 23 is a phase-2 (antigen-e,n,x) stable strain received from the Lister Institute of Preventive Medicine, London, by the courtesy of Dr B. A. D. Stocker. SJ 25 is a stable *1, 2* (phase-2) type recombinant obtained by transduction of the H_2 gene from *S. typhimurium* TM 2 to SL 23 (Iino, 1961 *a*).

Transduction was carried out with phage PLT 22 grown on the indicated host cultures. The general procedures of cultivation and transduction were according to the methods of Stocker, Zinder & Lederberg (1953) and Lederberg & Iino (1956). Bacterial motility was observed by darkground microscopy of hanging drops of broth. Clonal motility of bacteria was examined on semisolid medium (Stocker *et al.* 1953). The staining of flagella was by the method of Leifson (1951).

Specific flagellar antisera were prepared in the way described by Edwards & Ewing (1955). Antigen type was identified qualitatively by slide agglutination tests with antisera of titre 100, and when necessary qualitatively by tube agglutination (Kauffmann, 1954).

RESULTS

Association of wavelength interchange with phase variation in Salmonella typhimurium *SW* 577

When a broth culture of strain SW 577 is spread on semisolid media, it produces both compact colonies and swarms. Under the microscope in a hanging drop preparation, the bacteria from compact colonies were seen to rotate and aggregate together, while those from swarms move around and disperse. From the stained preparations it was found that the compact colonies were composed of bacteria with curly flagella (wavelength; $1 \cdot 1 \pm 0 \cdot 1 \mu$: amplitude; $0 \cdot 4 \pm 0 \cdot 05 \mu$) and the swarms of bacteria with normal flagella (wavelength; $2 \cdot 2 \pm 0 \cdot 1 \mu$: amplitude; $0 \cdot 5 \pm 0 \cdot 05 \mu$).

The subclones from a compact colony or a swarm dissociate repeatedly; that is, SW 577 is composed of two types of bacteria: those with normal flagella and those with curly flagella, and the two types interchange. The frequencies of the inter-changes between the two types are of the order of 10^{-4}/bacterium/division. This frequent to and fro change of flagellar shape in a culture resembles the phase variation in

flagellar antigen type (Stocker, 1949; Lederberg & Iino, 1956). Examination of the flagellar antigen of the normal and the curly clones demonstrated that the change in flagellar shape corresponded exactly with phase variation: in strain SW 577, clones with curly flagella were always in phase-1 (antigen-*i*) and clones with normal flagella were always in phase-2 (antigen-*1,2*) (Table 1).

Table 1. *Flagellar characters of* 50 *subclones derived from a broth culture of* Salmonella typhimurium *SW* 577

A broth culture of SW 577 was streaked out on a nutrient agar plate, the plate was incubated for 24 hr. at 37°, and flagellar characters were examined on 50 discrete colonies.

Number of subclones	Flagellar shape	H antigen type	Motility	Cells in liquid culture
26	Curly	*i*	Rotation	Aggregate
24	Normal	*1,2*	Translation	Disperse

Stability of curly character

The curly flagellar type in the *i*-phase of SW 577 rarely reverted to normal flagellar type. Because of the deficient motility of bacteria with curly flagella, such revertants could be detected as swarms on a plate of semi-solid medium containing anti-*1, 2* serum on which both curly *i* and normal *1, 2* clones produced only compact colonies. The reverted clones consisted of bacteria with flagella of normal wavelength, and were actively motile in both phases. The reversion rates to normal flagellar type from the curly phase-1 in strain SW 577 and in all the other curly mutants which will be described below were lower than 10^{-6}/division. So long as the curly genotype was maintained, the phenotypic expression in a curly flagellar phase was not modified under wide range of cultural conditions investigated: temperatures from 5° through 40°; in the range from pH 5·5 to 8·5; in broth, on nutrient agar or semisolid plates and in Davis' minimal or complete media (Lederberg, 1950). Staining of flagella by the method of Leifson gave convincing and reproducible figures of normal or curly types.

The genetic locus of a curly flagellar determinant

Transduction was carried out from a phase-2 culture of *Salmonella abony* (H-antigens *b*: *e,n,x*) strain CDC-103 to a phase-1 culture of SW 577 (curly-*i*: *1, 2*). Transductional clones producing rapidly spreading swarms on semi-solid plates containing anti-*1,2* serum were isolated. The antigen type and the shape of flagella of these isolated transductional clones are listed in Table 2. Among 100 clones tested, 42 had received the phase-2 antigen determinant of the donor, H_2^{enx}, but in phase-1 showed the same antigen, *i*, and curly flagella as the recipient; 56 clones had received both the phase-1 antigen determinant H_1^b and the determinant of the normal flagellar wavelength from the donor strain. The remaining two clones had acquired normal wavelength flagella, but neither of the H-antigens of the donor. These results indicate that the flagellar shape determinant was closely associated with H_1. The production of five *i*: *1,2* swarms in the control cultures suggests that two clones of the same type obtained from the transductional experiment were revertants of the curly flagella determinant, rather than recombinants between the antigen type determinant and the flagellar shape determinant.

Transduction was next carried out from a phase-1 culture of SW 577 to a phase-1 culture of an Ah_1-strain, *Salmonella typhimurium* SW 1061 (Iino, 1958, 1961 b). Ah_1 is a controller of H_1-activity and is closely linked to H_1; a strain which carries Ah_1^- cannot produce flagella, and consequently is non-motile, while it is in phase-1, but when in phase-2 it produces flagella and is normally motile. If the Ah_1^+ gene of SW 577 can be transduced separately from the curly determinant, normal i-type recombinants will be produced, and can be detected as swarms on semi-solid plates containing anti-$1,2$ serum. The result of transduction confirmed this expectation: 41 normal i-type recombinants grew from the transduction mixture, whereas only one grew in the control plates, on which the same amount of the SW 1061 cultures was incubated without the phage lysate of SW 577. It indicates that Ah_1 and the determinant of the curly flagella were separable by recombination, although Ah_1 was closely linked to H_1 with which the curly phase-1 determinant is closely associated.

Table 2. *Transduction from a phase-2 culture of* Salmonella abony $(b:e,n,x)$
to a phase-1 culture of S. typhimurium $SW 577$ $(curly-i:1,2)$

Recombinants were selected for on plates of semisolid medium containing anti-$1,2$ serum. Donor: 2×10^8 phage particles. Recipient: 5×10^7 bacteria.

	Flagellar antigens	Flagellar shape	Number of clones
Donor	$b:e,n,x$	Normal	—
Recipient	$i:1,2$	Curly in phase-1	—
Recombinants	$i:e,n,x$	Curly in phase-1	42
	$b:1,2$	Normal	56
	$i:1,2$	Normal	2
Revertants from control cultures	$i:1,2$	Normal	5

Curly flagella in phase-1 of Salmonella abortus-equi SL 23

Salmonella abortus-equi is a serotype which is generally stable in phase-2, antigen-e,n,x, and carries Vh_2^-, a gene which prevents the normal change of flagellar antigenic phase (Iino, 1958, 1961 a). The hidden phase-1, a-antigen type, is obtained by antiserum selection of rare H_2-inactive bacteria or by the introduction of an inactive H_2 gene by transduction. Transduction was carried out from a phase-1 culture of *S. typhimurium* TM 2 $(i:1,2)$ to a phase-2 culture of *S. abortus-equi* SL 23 $(a:e,n,x)$ on semisolid media containing anti-e,n,x serum, in the expectation of obtaining a stable a-type by the incorporation of an inactive $H_2^{1,2}$ gene. Recombinant swarms were detected after prolonged (3 days) incubation. Two among 35 swarms thus obtained spread normally. The remaining 33 spread very slowly and formed compact swarms. Flagellar shape was examined on the clones derived from both the normal and the compact swarms after staining by Leifson's method. The clones from the normal swarms were composed of bacteria with normal flagella while those from the compact swarms were composed of bacteria with curly flagella. The curvatures (wavelength and amplitude) of the normal and the curly flagella in SL 23 are the same as those in SW 577. One difference between the two curly types was that in liquid media phase-1 cells of SL 23 did not aggregate as remarkably

as those of SW577; consequently, the former produced compact swarms instead of compact colonies on a semisolid plate.

Normal flagellar character was transduced from TM2 to a curly-*a* transductional derivative of SL23. The 20 rapidly spreading swarms obtained on semisolid plates were all *i*-type. From these results it is inferred that, like SW577, SL23 has curly flagella in phase-1 and normal in phase-2 and that the curly determinant in phase-1 is closely associated with H_1.

Curly flagella in phase-2

Bacteria with curly flagella spread very slowly through semisolid medium. Therefore, when a culture with normal flagella is plated on such a medium, a curly mutant is expected to be detected as a dense spot on the faint sheet of motile bacteria. By the use of this method, 63 independently-occurring mutants which did not spread or spread very slowly were isolated from a stable *e*, *n*, *x*-type culture of SL23. Four of them, SJ30, SJ163, SJ164 and SJ165, were identified to be curly mutants. Four others were paralysed mutants, and the remaining 55 were *Fla*⁻. The same

Table 3. *Transductions from the strains of* Salmonella *with normal flagella to those with curly flagella in phase-2*

All of the recipient strains are phase-2 monophasic (Vh_2^-) types. Recombinants were selected for on plates of semisolid medium, on which only the bacteria with normal flagella in phase-2 could produce swarms. Swarms on the control culture were produced by revertants of curly flagellar determinant. Donor: 3×10^8 phage particles; recipient: 1×10^8 bacteria, in each combination.

		Antigen types of swarms from			
		Transduction mixture		Control culture	
Donor	Recipient	*1,2*	*e,n,x*	*1,2*	*e,n,x*
Phase-2 culture	SJ30 curly-*e,n,x*	113	4	0	6
of TM2 (*i:1,2*)	SJ163 curly-*e,n,x*	75	11	0	19
	SJ164 curly-*e,n,x*	80	2	0	4
	SJ165 curly-*e,n,x*	108	6	0	11
SL23 (*:e,n,x*)	SJ167 curly-*1,2*	16	109	18	0
	SJ168 curly-*1,2*	2	90	2	0

selection method was applied to a *1,2*-type culture of SJ25, which is a recombinant obtained by the transduction of the H_2 gene from *Salmonella typhimurium* strain TM2 to SL23, and two curly mutants, SJ167 and SJ168, were isolated from it. The transduction of the determinant of normal flagellar shape to these curly mutants was always associated with the transfer of the phase-2 antigen determinant H_2 (Table 3). One remarkable feature of these transductions is that trails were not produced at all on the selective media (see Lederberg, 1956; Stocker, 1956). This suggests that the normal type is not completely dominant over the curly type.

As both SL23 and SJ25 are stable phase-2 monophasic types, each carrying a hidden curly phase-1 determinant, no direct evidence has been obtained to exclude the possibility that the newly acquired mutant genes may affect the flagellar shape in phase-1 as well as in phase-2. Nevertheless, it is probable, by analogy with the curly determinant in phase-1, that the function of these phase-2 curly determinants is also phase-specific.

Flagellar antigens of the curly mutants

The close association of the curly flagella determinants and the H-antigen type determinants in each phase urged us to the detailed examination of the antigenicity of normal and curly flagella. Anti-i sera were obtained against both TM2 (normal-i flagella) and SW577 (curly-i flagella), and the agglutination titres of the anti-i sera to the i-phases of these strains were examined before and after reciprocal absorption (Table 4). The results showed that each serum had the same titre on both normal-i (TM2) and curly-i (SW577) suspensions, and that each serum could be completely absorbed by the heterologous suspension. That is, the i-antigens of curly-i and normal-i flagella behave identically in the serum agglutination experiment. Parallel results were obtained with SL23 (normal-e,n,x flagella) and one of its curly mutants, SJ30 (curly-e,n,x flagella).

Table 4. *Tube agglutination test of the H-antigens in normal and curly flagella*

The method followed was that of Kauffmann (1954). 0, absence of agglutination at 1 to 8; —, not tested.

Antiserum against	Absorbed by	Titre against			
		TM2 i	SW577 curly-i	SL23 e,n,x	SJ30 curly-e,n,x
TM2 i	—	16,400	16,400	—	—
	TM2 i	0	0	—	—
	SW577 curly-i	0	0	—	—
SW577 curly-i	—	22,000	22,000	—	—
	TM2 i	0	0	—	—
	SW577 curly-i	0	0	—	—
SL23 e,n,x	—	—	—	12,000	12,000
	SL23 e,n,x	—	—	0	0
	SJ30 curly-e,n,x	—	—	0	0
SJ30 curly-e,n,x	—	—	—	16,400	16,400
	SL23 e,n,x	—	—	0	0
	SJ30 curly-e,n,x	—	—	0	0

Transductions between curly flagellar mutants

All the curly flagellar mutants so far obtained revert to the normal type at frequencies which would obscure a low frequency of transduction. Consequently, it is difficult to obtain clear evidence of recombination between curly type determinants of two strains with the same antigen type. In cases where the antigen types of the two mutants are different, we may conclude that a normal type which has the H-antigen of the donor strain in transduction is produced by a recombination between two curly flagella determinants.

In order to get the recombination between the curly flagellar determinant, transduction was carried out from curly-i type of SW577 to curly-a type of SL23, and from curly-e,n,x type of SJ30 to curly-$1,2$ type of either SJ167 or SJ168. In the first combination, a phase-2 culture was used for the preparation of lysate and the transduction of $H_2{}^{1,2}$ was scored as a control: when a phase-2 culture is used as a donor, transduction of H_1 and H_2 are expected to be equally common (Lederberg

& Iino, 1956). In transductions from SJ30 (curly-*e*,*n*,*x*), SL23 (normal-*e*,*n*,*x*) was used as the donor in control experiments. So far, recombinants with normal flagella and with the H-antigens of the donor have not been obtained from any combination, while in each control combination over 100 transductional recombinants were obtained. This suggests that the mutant sites of the two phase-1 curly types are very closely linked or identical; and similarly that the sites of the three phase-2 curly mutants are very closely linked or identical.

DISCUSSION

The present investigation demonstrated that curly flagellar shape is H-antigen phase-specific in salmonella. The frequent dissociation of a diphasic strain of *Salmonella typhimurium* SW577 to normal and curly flagellar types (Leifson & Hugh, 1953) represents antigenic phase variation. In addition to SW577, Leifson (1951) described the dissociation of normal and curly flagellar types in another salmonella strain, *S. wichita* SJ64. Serological examination of the dissociated subclones of this strain has now shown that the two curvature types are likewise associated with antigenic phases: curly in phase-1 (*d*-antigen) and normal in phase-2 (Z_{37}-antigen).

Genetic determinants of the curly flagella are closely associated with the determinants of H-antigen type, H_1 and H_2 in phase-1 and phase-2 respectively. The flagella of the genus *Salmonella* are composed of a fibrous protein called flagellin (Astbury *et al.* 1955; Ambler & Rees, 1959). The H-antigen type of their flagella is presumed to reflect a specificity of flagellin molecules. The experiment of Kerridge (1959) on flagellar regeneration (see Introduction) suggested that flagellar curvature is also a manifestation of a chemical composition of flagellin. Chi-phage is a strain of bacteriophages which can attack only motile salmonella (Rakieten & Bornstein, 1941). Like the curly flagellar character, one type of resistance to this bacteriophage has been found to be phase-specific and associated with certain antigen types, antigens *g*,..., which are determined by the H_1 locus (Meynell, 1961); certain flagellins (or parts of them) are presumably the receptor of this phage. From these primacies of H_1 and H_2 in the determination of the specificity of flagella, it is inferred that H_1 and H_2 are the primary structural determinants of flagellin; a mutation in one of these genes produces an altered configuration of the corresponding flagellin, presumably through a change in its amino acid sequence, resulting in a change in antigen type, a modification of the flagellar shape or failure of either flagellin synthesis or flagellar morphogenesis.

For the synthesis of flagella, several *Fla* genes, as well as the H_1 and H_2 genes, are required (Stocker *et al.* 1953). These *Fla* genes are however not phase-specific and, as far as investigation has shown, they are not concerned with the specificity of flagella. The only gene which has been known to control a specificity of flagellin in both phases is that which determines the presence of *N*-methyl-lysine in flagellin (Stocker, McDonough & Ambler, 1961). Its function has been presumed to be not to serve as a primary structural determinant of flagellin but to control the production of a methylator of lysine in already made flagellin molecules.

The failure to obtain normal type recombinants from transductions between curly flagella mutants suggests that in each phase the sites of mutation in the various

curly type mutants tested are very closely linked or identical. As demonstrated by Kerridge (1959), curly flagella are produced when phenylalanine is replaced by p-fluorophenylalanine during flagellar regeneration. Therefore it seems quite possible that the curly flagella of curly mutants result from replacement of a particular amino acid in flagellin by some other; such mutants might occur by mutation of a specific site in H_1 or H_2. The comparative chemical analysis of the flagellins from normal and curly-mutant flagella is under investigation.

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A Neutralization Test for Trachoma and Inclusion Blennorrhoea Viruses Grown in HeLa Cell Cultures

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SUMMARY

A neutralization test for the viruses of trachoma and inclusion blennorrhoea grown in HeLa cell cultures is described. Antiserum titres, which are independent of virus concentration over a wide range, can be accurately and reproducibly estimated. Limited cross-neutralization tests indicate a close antigenic relationship between one strain of trachoma virus and two strains of inclusion blennorrhoea.

INTRODUCTION

Since the isolation of trachoma virus by T'ang, Chang, Huang & Wang (1957) similar agents have been isolated from patients with trachoma and inclusion blennorrhoea in at least thirteen countries. There are few serological tests for the exploration of these agents. In addition to its use as an index of immune response, a neutralization test might have important applications in the study of strain differences. An accurate titration method in cell culture (Furness, Graham & Reeve, 1960) now permits quantitative studies of neutralization *in vitro*. The present paper describes a neutralization test for trachoma and inclusion blennorrhoea viruses grown in cell culture.

METHODS

Cell cultures. HeLa cells were grown in 250 ml. Pyrex feeding bottles containing 10 ml. medium, and for virus titrations, in Leighton tubes containing coverslips and 2 ml. medium. The medium consisted of Hank's saline solution containing 5 % (v/v) calf serum, 10 % (v/v) human serum, and 0·5 % (w/v) lactalbumin hydrolysate, adjusted to pH 7·2–7·4 with 1·4 % (w/v) $NaHCO_3$.

Viruses. The TE 55 strain of trachoma virus (T'ang *et al.* 1957), the LB 1 strain of inclusion blennorrhoea (Jones, Collier & Smith, 1959) and LB 4 strain of inclusion blennorrhoea (Jones & Collier, unpublished) were used.

Virus suspensions. For immunization infected chick embryo yolk sacs were homogenized with sucrose + potassium glutamate (SPG) solution (Bovarnik, Miller & Snyder, 1950) to give a 20 %, w/v, suspension. After low-speed centrifugation the virus was deposited in a high-speed angle centrifuge at 4° and resuspended in SPG solution to two-fifths of the original volume. The infective titre of the suspensions was about 10^5 egg infectious doses/ml. (EID 50/ml.). The suspensions were stored at −70°.

HeLa cell-grown virus suspensions were prepared from infected monolayers of

HeLa cells grown in Pyrex feeding bottles. Three days after inoculation the cells from each bottle were suspended in 10 ml. medium and disrupted with an MSE Mullard ultrasonic disintegrator with a power output of 60 W. at 20 kc./sec. The virus was deposited in the high-speed angle centrifuge at 4° and resuspended in 1 ml. SPG solution.

For titration, infected HeLa cells from each Pyrex bottle were suspended in 10 ml. medium, disrupted as above, and diluted in medium. Cell-cultured virus was used immediately after preparation.

Antisera. Rabbits were immunized by weekly intravenous injections of live virus, receiving either six 1 ml. injections of yolk-sac-cultured virus or twelve 1 ml. injections of cell-cultured virus. They were bled 2 weeks after the final injection; the sera were stored at −20°. Sera were heated at 56° for 30 min. before use.

Neutralization test. A high concentration of serum was required to prevent heat inactivation of the virus. Accordingly, twofold dilutions of antiserum were made in normal rabbit serum and 0·5 ml. amounts were mixed with equal volumes of virus suspension. After incubation at 37° for 30 min., four Leighton tubes were each inoculated with 0·2 ml. of each serum + virus mixture to assess the number of inclusion forming units (IFU; Furness *et al.* 1960). Control tubes were inoculated with virus that had been incubated with normal rabbit serum or antiserum against normal yolk sac. The degree of neutralization was determined by comparing the number of IFU/ml. in the antiserum + virus mixtures with that of the controls.

RESULTS

Neutralization with undiluted antisera

Undiluted serum neutralized 90–100 % of virus when tested against concentrations ranging from $3·7 \times 10^7$ to $3·2 \times 10^4$ IFU/ml. (Table 1). This result suggests that neutralization was independent of virus concentration in the presence of excess antibody. Although the antiserum titres were low, neutralization appeared to be specific, since similar dilutions of control serum did not inhibit virus growth.

Neutralization with diluted antiserum

Figure 1 shows the neutralization of LB1 virus with sera prepared against both egg- and HeLa-cell-cultured antigens. Each point represents the mean of the infected cell counts in 4 replicate tubes inoculated with a given virus + serum mixture. Antiserum R197, prepared against LB1 virus cultured in HeLa cells, was titrated on four occasions against concentrations of virus ranging from $6·7 \times 10^4$ to $8·4 \times 10^5$ IFU/ml. The results of serum dilutions 1/32 and 1/64 are incomplete since some tubes were lost because of bacterial contamination. Antiserum R261, prepared against egg-cultured LB1 virus, was similarly titrated on two occasions; the infective titres of the virus suspensions were, respectively, $3·0 \times 10^5$ and $3·2 \times 10^4$ IFU/ml.

With serum dilutions which neutralized less than 75 % of virus, inhibition was directly proportional to serum dilution (Fig. 1). It is therefore convenient to express neutralization titres in terms of a 50 % end-point: since the virus assay has a standard error of ± 10 % (Furness *et al.* 1960), a 50 % decrease of virus titre is highly significant. Repeat titrations of both sera gave similar end-points; a 1/32
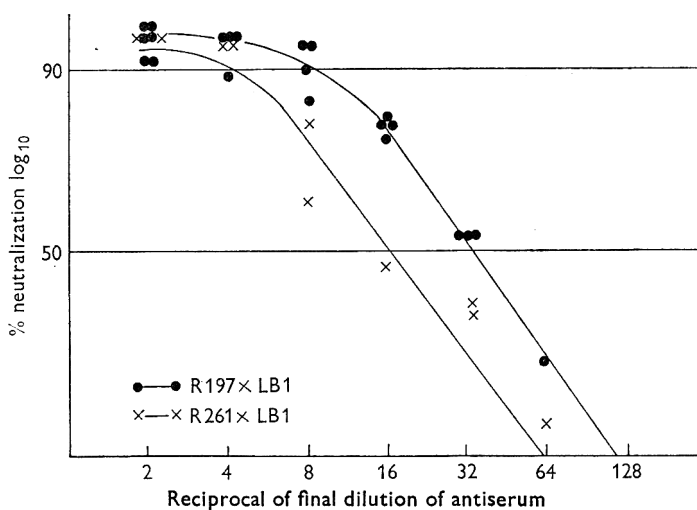
Fig. 1. Titration of 2 anti-LB1 rabbit sera against homologous virus. Each point is the mean of four replicates. Serum R197 (black dots) was titrated on four occasions. Serum R261 was titrated on two occasions.

dilution of antiserum R197 neutralized 50 % of virus over a 12-fold range and a 1/16 dilution of antiserum R261 over a 10-fold range of virus concentration.

Cross-neutralization. Experiments with single antiserum dilutions showed that the LB1 and the LB4 strains of inclusion blennorrhoea were neutralized by an antiserum prepared against the TE55 strain of trachoma virus (Table 1).

Table 1. *Neutralization of trachoma and inclusion blennorrhoea viruses in HeLa cell cultures*

Rabbit antiserum	Final dilution	Strain of virus neutralized	Before neutralization	After neutralization	Degree of neutralization (%)
R143 anti-TE55	1/2	TE55	$3 \cdot 7 \times 10^7$	$2 \cdot 6 \times 10^6$	93
		TE55	$3 \cdot 7 \times 10^6$	$* < 7 \cdot 0 \times 10^3$	> 99
		TE55	$8 \cdot 4 \times 10^5$	$< 7 \cdot 0 \times 10^3$	> 99
R261 anti-LB1	1/2	LB1	$3 \cdot 7 \times 10^5$	$< 7 \cdot 0 \times 10^3$	> 98
		LB1	$3 \cdot 4 \times 10^4$	$< 7 \cdot 0 \times 10^3$	> 80
		LB1	$3 \cdot 2 \times 10^4$	$4 \cdot 4 \times 10^3$	88
R197 anti-LB1	1/2	LB1	$8 \cdot 4 \times 10^5$	$1 \cdot 2 \times 10^4$	98
		LB1	$8 \cdot 0 \times 10^5$	$3 \cdot 0 \times 10^4$	96
		LB1	$7 \cdot 4 \times 10^5$	$< 7 \cdot 0 \times 10^3$	> 99
		LB1	$6 \cdot 7 \times 10^4$	$< 7 \cdot 0 \times 10^3$	> 90
		LB1	$5 \cdot 0 \times 10^4$	$< 7 \cdot 0 \times 10^3$	> 86
R143 anti-TE55	1/2	LB1	$3 \cdot 7 \times 10^5$	$< 7 \cdot 0 \times 10^3$	> 98
	1/10	TE55	$8 \cdot 4 \times 10^5$	$4 \cdot 0 \times 10^5$	62
	1/8	LB4	$1 \cdot 5 \times 10^5$	$5 \cdot 9 \times 10^4$	60
	1/8	LB4	$9 \cdot 4 \times 10^4$	$3 \cdot 7 \times 10^4$	60

* Virus suspension containing less than $7 \cdot 0 \times 10^3$ IFU/ml. cannot be assayed by the technique used.

DISCUSSION

Bell, Snyder & Murray (1959) showed that immunization protected mice against the toxic effects of an intravenous dose of live trachoma virus. This test revealed at least two antigenically distinct strains of trachoma virus from Saudi Arabia and Egypt. This test can, however, be applied only to strains that are toxic for mice; furthermore, the toxic effect cannot be neutralized *in vitro*. Bernkopf (1959) found that some strains of trachoma virus induced specific pulmonary lesions in mice when instilled intranasally, their capacity to do this being neutralized by preliminary incubation with serum from immunized rabbits.

The present paper describes the first *in vitro* neutralization test for the viruses of trachoma and inclusion blennorrhoea grown in cell culture, by which antiserum titres can be accurately estimated. In previously described neutralization tests with these and other viruses of the psittacosis-lymphogranuloma group, virus is titrated against a constant dilution of antiserum (see review by Wenner, 1958). It is not practicable to use a constant amount of virus in these tests because so far it has proved impossible to store cell-cultured viruses without loss of infectivity. Nevertheless, the 50 % neutralization end-point is independent of virus concentration over a wide range, and the yield from a 250 ml. bottle culture 2 or 3 days after inoculation is sufficient for a neutralization test, without a determination of its exact titre. Antiserum titres at this degree of neutralization are sufficiently high to permit cross-absorption tests. Limited cross-neutralization tests indicate a close antigenic relationship between a Chinese strain of trachoma and two London strains of inclusion blennorrhoea.

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