THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

VOLUME 51, 1968

CAMBRIDGE
AT THE UNIVERSITY PRESS
1968

PUBLISHED BY THE CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W.1
American Branch: 32 East 57th Street, New York, N.Y. 10022

Printed in Great Britain at the University Printing House, Cambridge

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Part 1 *issued* April 1968

Part 2 *issued* April 1968

Part 3 *issued* May 1968

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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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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*, 1962. (Cambridge University Press.)

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

BACTERIA. Author's preferences 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.

J. gen. Microbiol. (1968), **51**, 1–16
Printed in Great Britain

Fimbriation, Pellicle Formation and the Amount of Growth of Salmonellas in Broth

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(*Accepted for publication* 29 *August* 1967)

SUMMARY

In aerobic static broth cultures grown at 37°, salmonellas of genotype Fim(1)$^+$, i.e. bearing type-1 (mannose-sensitive, haemagglutinating) fimbriae, formed a surface pellicle consisting of densely packed bacteria shortly after the cessation of logarithmic growth at about 6 hr, and then, during the next 24 hr, underwent a large secondary phase of growth. Salmonellas of genotype Fim(2)$^+$, i.e. bearing type-2 (non-haemagglutinating) fimbriae, and most Fim$^-$ (non-fimbriate) salmonellas did not form a pellicle and gave only slight post-logarithmic growth; after 24–72 hr the amount of their growth, i.e. bacterial concentration estimated turbidimetrically, was only one-third to one-half that of comparable type-1 fimbriate bacteria. Fim$^-$ strains of *Salmonella typhimurium* differed from most other Fim$^-$ salmonellas by forming a pellicle and undergoing an associated secondary phase of growth, but they gave these effects 24–48 hr later than did the Fim(1)$^+$ strains. A pellicle was not formed by any organism either in static cultures incubated anaerobically or in cultures aerated by continuous shaking, and in these two sets of conditions the course and amount of growth were the same for the Fim(1)$^+$ as for the Fim$^-$ bacteria. It is concluded that the large secondary phase of growth shown by the Fim(1)$^+$ bacteria in aerobic static broth results from the free availability of atmospheric oxygen to the bacteria growing in the pellicle.

The presence of 0·2% (w/v) of the haemagglutination-inhibiting sugar, α-methylmannoside, which was not utilized within 6 days, delayed by more than 24 hr the formation of a pellicle and the onset of the secondary phase of growth in aerobic static cultures of the Fim(1)$^+$ strain LT2 of *Salmonella typhimurium*. Another haemagglutination-inhibiting sugar, D-mannose, was utilized within 12 hr and delayed pellicle formation by only 2–3 hr in cultures of strain LT2, but it caused a prolonged (> 24 hr) delay in pellicle formation in cultures of two non-mannose-utilizing mutants of strain LT2. D-glucose and L-sorbose, which do not inhibit haemagglutination, did not delay pellicle formation. These findings, together with the failure of Fim(2)$^+$ bacteria to form a pellicle, suggest that the property of type-1 fimbriae that promotes early pellicle formation is the same as that responsible for the mannose-sensitive haemagglutinating activity.

INTRODUCTION

There is still no definite knowledge of the function of type-1 fimbriae, the small numerous filamentous appendages of bacteria which were first distinguished from flagella and capsular material by Houwink & van Iterson (1950). These authors suggested that fimbriae serve as organs of adhesion, and the presence of adhesive

properties in fimbriae was later confirmed by the findings that fimbriate bacteria adhere to and agglutinate red blood cells (Duguid, Smith, Dempster & Edmunds, 1955) and that they tend to grow closely adherent to one another in the form of a pellicle on the surface of aerobic static broth cultures (Duguid & Gillies, 1957). The property of early, fimbriae-dependent pellicle formation enables fimbriate-phase cultures of *Shigella flexneri* to attain a much greater amount of growth than is attained by non-fimbriate-phase cultures of the same organism, and this advantage is probably due to the free access to atmospheric oxygen enjoyed by the fimbriate bacteria when growing in the pellicle (Duguid & Wilkinson, 1961).

Shigellas, however, are strict parasites and under natural conditions do not grow in stagnant poorly aerated aqueous habitats where pellicle formation might be advantageous. Saprophytic enterobacteria which do live in such habitats, e.g. species of the genera *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus* and *Serratia*, are also fimbriate, and possibly therefore might benefit from the ability to form a pellicle. However, most of these saprophytes differ from the shigellas in being motile, and motile enterobacteria, including salmonellas, have been shown by Baracchini & Sherris (1959) to migrate with positive aerotaxis towards the oxygenated surface layer in a poorly oxygenated liquid medium. Since it seemed possible that motility might either promote or inhibit the formation of a pellicle by fimbriate bacteria, and that aerotaxis might substitute for pellicle formation as a means of procuring access of the bacteria to atmospheric oxygen, we thought it important to discover whether fimbriation has the same influence in determining pellicle formation and the amount of growth in motile bacteria as it has in non-motile ones.

Salmonellas were selected for these studies because they include genotypically fimbriate (Fim^+) and genotypically non-fimbriate (Fim^-) strains that may be compared with one another. Moreover, Fim^- strains of *Salmonella typhimurium* are readily transduced to the Fim^+ genotype with phage P22 (Duguid, Old & Hume, 1962; Old, 1963), and since the amount of DNA transferred in transduction is small, a Fim^- strain and its Fim^+ transductant are likely to resemble each other very closely. Differences in growth behaviour may, therefore, be attributed more reliably to the difference in fimbriation when a Fim^- strain is compared with its Fim^+ transductant than when it is compared with an unrelated wild-type Fim^+ strain.

Most fimbriate salmonellas resemble fimbriate escherichias and shigellas in bearing fimbriae that posses a haemagglutinating activity specifically sensitive to inhibition by D-mannose and α-methylmannoside. These mannose-sensitive HA^+ fimbriae are designated 'type 1' in the classification of Duguid, Anderson & Campbell (1966) and the genotype of the bacteria bearing them is given as $Fim(1)^+$. A minority of fimbriate salmonellas bear a different, non-haemagglutinating (HA^-) type of fimbriae, designated 'type 2' and their genotype is given as $Fim(2)^+$. Tests were made with some $Fim(2)^+$ strains to determine whether or not the HA^+ character was essential for the promotion of pellicle formation. To the same end, observations were made of the effect of the presence of mannose and α-methylmannoside on pellicle formation by $Fim(1)^+$ bacteria.

METHODS

Organisms. The strains used in the main experiments are listed in Table 1. Those of *Salmonella typhimurium* included 4 wild-type Fim(1)$^+$ strains, 7 wild-type Fim$^-$ strains, and 5 Fim(1)$^+$ strains that had been derived from 5 of the Fim$^-$ strains by transduction with phage. (As shown in Table 1, the 'wild-type' strains SL497, SW573 and SL272 were variants that differed from parental wild-type strains in characters other than fimbriation.) Transduction of the Fim(1)$^+$ character was done by mixing the Fim$^-$ acceptor culture with a lysate of phage P22 propagated on one of the Fim(1)$^+$ strains, SW375 and SL497; the Fim(1)$^+$ transductants were selected by serial subculture for 48 hr periods in aerobic static broth. Phage-types were determined by Dr E. S. Anderson according to the scheme of Callow (1959).

Table 1. *Strains of Salmonella used in the main experiments*

Serotype and no. of strain	Origin (and supplier)	Phage-type	Fimbriation genotype (and type of fimbriae)	Flagella	Motility
S. typhimurium					
LT2	Phage-type-2 strain of Lilleengen (1948) (Dr B. A. D. Stocker)	4	+ (1)	+	+
S206	Patient (Dr Helen A. Wright)	2	+ (1)	+	+
SW375	Strain Q1 of Boyd (1956) (Dr B. A. D. Stocker)	1	+ (1)	+	+
SL497	Streptomycin-resistant mutant from LT2 (Dr B. A. D. Stocker)	.	+ (1)	+	+
SW573	Variants of strain of Friewer &	U165	−	−	−
SW578	Leifson (1952) (Dr B. A. D.	13	−	+	−
SL272	Stocker)	13	−	+	+
SL272F	Fim$^+$ transductant from SL272	.	+ (1)	+	+
S575	Patient (Dr Helen A. Wright)	13	−	+	+
S575F	Fim$^+$ transductant from S575	.	+ (1)	+	+
S625	Fowl (Dr J. E. Wilson)	14	−	+	+
S625F	Fim$^+$ transductant from S625	.	+ (1)	+	+
S635	Fowl (Dr J. E. Wilson)	13	−	−	−
S635F	Fim$^+$ transductant from S635	.	+ (1)	−	−
M7471	Patient (Dr E. S. Anderson)	1	−	+	+
M7471F	Fim$^+$ transductant from M7471	.	+ (1)	+	+
SL970	Non-mannose-fermenting mutants	.	+ (1)	+	+
SL1031M	from LT2 (Dr B. A. D. Stocker)	.	+ (1)	+	+
S. choleraesuis					
NCTC5364	National Collection of Type Cultures (NCTC)	.	−	+	+
NCTC5738	NCTC		+ (1)	+	+
S. paratyphi B					
NCTC5707	NCTC	.	+ (2)	+	+
NCTC5707HA$^+$	HA$^+$ transductant from NCTC5707	.	+ (1)	+	+
NCTC8390	NCTC	.	+ (1)	+	+

HA$^+$ = haemagglutinating.

Two non-mannose-fermenting mutant strains derived from strain LT2 of *Salmonella typhimurium* were kindly supplied by Dr B. A. D. Stocker. Strain SL970, which lacks the enzyme phospho-mannose-isomerase, had been derived as mutant EP9 by Dr Mary J. Osborn (Yeshiva University, New York). Strain SL1031M is a motile trans-

ductant of strain SL1031, a mutant (RS60) derived by Dr R. G. Wilkinson (Lister Institute of Preventive Medicine, London).

Single wild-type Fim(1)$^+$ and Fim$^-$ strains of *Salmonella choleraesuis* were examined, also 3 strains of *S. paratyphi B*: a wild-type Fim(1)$^+$ strain, a wild-type Fim(2)$^+$ strain and a Fim(1)$^+$ transductant derived from the Fim(2)$^+$ strain. Less detailed observations were made with many other salmonellas selected from the series of Duguid *et al.* (1966).

Culture media. Nutrient broth contained: Oxoid bacteriological peptone, 1 g.; Oxoid Lab-Lemco meat extract, 1 g.; NaCl, 0·5 g.; water, 100 ml. Phosphate-buffered broth, used for cultures with sugars, was nutrient broth buffered at pH 7·0 by the addition of KH_2PO_4, 0·35 g., and Na_2HPO_4, 0·65 g., per 100 ml.

Incubation. This was at 37° and three methods were used: (1) Aerobic static cultures were grown in 10 ml. volumes of broth in cottonwool-stoppered test-tubes (15 × 1·5 cm.) incubated in air, without movement or disturbance. (2) Anaerobic static cultures were grown in 10 ml. volumes of broth in similar tubes incubated without movement in a McIntosh & Fildes anaerobic jar filled with hydrogen. (3) Aerobic shaken cultures were grown in 40 ml. volumes of broth in 1 l. bottles which were incubated in a machine that continuously rotated them at 20 rev./min. around their long axis, held horizontally.

Examination for pellicle. The pellicle on the surface of the broth was often thick enough to be easily seen when the undisturbed tube of culture was viewed from the side or from above. Sometimes, however, particularly in the early stages of growth, it was so thin and transparent as to be invisible under these conditions of viewing. Routinely, therefore, the tubes were viewed from the side while they were gently shaken in such a way as to cause any pellicle present to float a little way up the wall of the tube, where it became clearly visible.

Measurement of amount of growth. This was done turbidimetrically either with a Spekker absorptiometer or, at 530 mμ, with a Unicam spectrophotometer. Dense cultures were first diluted with sterile broth to give a reading of less than 0·4 and the reading obtained was multiplied by the dilution factor to give the value for the undiluted culture. In all strains examined in cultures over 6 hr old, a turbidity value of 1·0 was equivalent to about 10^9 bacteria/ml.

Haemagglutination tests. The presence and abundance of type-1 fimbriate bacteria in a culture was estimated by the presence and strength of haemagglutinating activity. A drop of dense bacterial deposit obtained by centrifugation of the broth culture was mixed for 10 min. with a drop of a 3% (v/v) suspension of washed guinea-pig red blood cells (Duguid *et al.* 1955). A test was also made with a drop of uncentrifuged broth culture, or of deposited bacteria resuspended in saline to the original concentration of the culture. When a positive reaction was obtained with this reduced concentration of bacteria, it was concluded that the culture was richly fimbriate.

Experimental procedure. Each culture was inoculated with about 10^7 bacteria taken either from a colony grown for 24 hr on nutrient agar, or after it had been thoroughly mixed, from an aerobic static broth culture grown for 24 or 48 hr. All strains, except *Salmonella choleraesuis* strain NCTC5738, gave fimbriate-phase cultures in aerobic static broth whichever of these types of inoculum was used. Strain NCTC5738 gave a non-fimbriate-phase culture when the inoculum was from agar. Fim$^+$ and Fim$^-$ strains were cultured in parallel, each strain by each of the three methods of incubation.

For each method of incubation, a series of tubes or bottles of broth were given equal inocula. After incubation of the series for each different interval of time, a different tube, or bottle, was taken from the incubator and examined for pellicle. The culture was then homogenized by mixing with a pipette until any pellicle or deposit was completely dispersed. The organisms were killed by the addition of 0·25% (v/v) formaldehyde and the amount of growth was measured turbidimetrically. Finally, the culture was centrifuged and tested for haemagglutinating activity.

RESULTS

Pellicle formation in aerobic static broth

Examinations for pellicle formation in aerobic static broth were made at intervals during growth for 96 hr at 37° on the series of salmonellas in 149 serotypes studied for fimbriation by Duguid *et al.* (1966). The results for the majority of strains in representative serotypes are summarized in Table 2. All the Fim(1)$^+$ strains, except for a few

Table 2. *Pellicle formation by salmonellas of different fimbriation genotypes in aerobic static broth cultures incubated at 37° for 96 hr*

The results are those observed for the majority of the strains in each serotype.

Fimbriation genotype	Serotype	Pellicle formation (and time in hr of first appearance of pellicle) when inoculum was from a culture	
		On agar*	In broth†
Fim (1)$^+$ (i.e. Fim$^+$ HA$^+$)	typhimurium	+ (8–12)	+ (6–8)
	paratyphi B	+ (8–12)	+ (6–8)
	choleraesuis	+ (8–12)	+ (6–8)
	typhi	+ (8–12)	+ (6–8)
	Most other serotypes	+ (8–12)	+ (6–8)
Fim (2)$^+$ (i.e. Fim$^+$ HA$^-$)	paratyphi B	–	–
	gallinarum	–	–
	pullorum	–	–
Fim$^-$	typhimurium (motile)	+ (36–72)	+ (18–48)
	typhimurium (non-motile)	–	–
	paratyphi A	–	–
	paratyphi B	–	–
	paratyphi C	–	–
	choleraesuis	–	–
	typhi	–	–
	pullorum	–	–

* Inoculum was taken from a colony on a nutrient agar plate incubated for 24 hr. Some Fim(1)$^+$ strains produced pellicles much more slowly when inoculated from non-fimbriate-phase cultures on agar. *S. typhimurium* LT 2 usually did so only after 12–24 hr and *S. choleraesuis* NCTC 5738 after 48–96 hr.

† Inoculum was taken from an aerobic static broth culture incubated for 48 hr.

that were very poorly fimbriated, formed a visible surface pellicle covering the whole surface of the broth; a thin but complete pellicle was usually present after 6–10 hr. A pellicle was not formed within 96 hr by the Fim(2)$^+$ strains, e.g. of *Salmonella paratyphi B*, *S. gallinarum* and *S. pullorum*, or by the Fim$^-$ strains in most of the serotypes that included such strains, e.g. *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. choleraesuis*, *S. typhi* and *S. pullorum*. Fim$^-$ strains of *S. typhimurium* differed from

other Fim$^-$ salmonellas by forming a pellicle, but they did so only after a much longer period of incubation, usually between 24 and 72 hr, than was required for pellicle formation by Fim(1)$^+$ organisms (6–10 hr). Rare Fim$^-$ strains of *S. typhimurium* were non-motile, being non-flagellate (SW 573, S635) or having 'paralysed' flagella (SW 578), and these strains usually failed to form a pellicle. Fim(1)$^+$ transductants derived from Fim$^-$ strains of *S. typhimurium* and from a Fim(2)$^+$ strain of *S. paratyphi B* resembled wild-type Fim(1)$^+$ strains in producing a pellicle at an early stage in culture.

The pellicle in Fim(1)$^+$ cultures appeared at a slightly earlier stage (6–8 hr) when the inoculum was from a 24 hr or 48 hr broth culture containing mainly, e.g. over 50%, fimbriate-phase bacteria than when it was from an agar-grown colony containing mainly non-fimbriate-phase bacteria (usually 8–12 hr). Most Fim(1)$^+$ salmonellas change rapidly from the non-fimbriate to the fimbriate phase when cultured in aerobic static broth, and this appears to be the reason why non-fimbriate-phase inocula usually produced a pellicle in 8–12 hr. A few Fim(1)$^+$ strains, however, were exceptionally slow in changing to the fimbriate phase and in forming a pellicle in cultures inoculated from agar; e.g. *S. typhimurium* strain LT 2 showed a pellicle usually only after 12–24 hr. in such conditions and *S. choleraesuis* NCTC 5738 did so only after 48–96 hr.

A visible pellicle was not formed by any organism in static broth cultures incubated under completely anaerobic conditions.

Amount of growth related to fimbriation and pellicle formation

Growth curves for each of the three conditions of incubation were determined for several representative Fim(1)$^+$, Fim(2)$^+$ and Fim$^-$ strains. The amount of growth (bacterial concentration) was measured turbidimetrically after the culture had been homogenized, and since this procedure involved the dispersion of pellicle, the observation at each succeeding interval of time was made on a different, replicate, culture which had been incubated undisturbed until the time of examination. Experiments with typical findings are illustrated in the figures. Figure 1 compares the growth curves of Fim$^-$ and Fim(1)$^+$ strains of *Salmonella choleraesuis*, Fig. 2 compares those of a Fim$^-$ strain of *S. typhimurium* and its Fim(1)$^+$ transductant and Fig. 3 compares those of a Fim(2)$^+$ strain of *S. paratyphi B* and its Fim(1)$^+$ transductant.

During the logarithmic phase of growth, which was completed in the first 6 hr, the rate of growth was practically the same for Fim(1)$^+$, Fim(2)$^+$ and Fim$^-$ organisms and for each of the different methods of incubation: aerobic shaken, aerobic static and anaerobic static. Thereafter, the growth curves of the cultures differed with the method of incubation and also, in the case of the aerobic static cultures, with the organism's state of fimbriation.

In anaerobic static and aerobic shaken cultures the growth curves of the Fim(1)$^+$ organisms were almost identical with those of the Fim$^-$ ones. The amount of growth reached its maximum value by about 8 hr, after which it remained nearly constant. The maximum value, as turbidity reading, in the anaerobic cultures was usually between 0·25 and 0·3 (i.e. $0{\cdot}25–0{\cdot}3 \times 10^9$ bacteria/ml.), whereas that in the aerobic shaken cultures was much higher, usually between 4·0 and 8·0. The reason why the bacteria gave about 20-fold greater growth under aerobic shaken than under anaerobic conditions was probably that they were able to obtain proportionately this much more energy by the oxidation than by the fermentation of the nutrient substrates in the broth.

In aerobic static cultures the end of the logarithmic phase of growth at about 6 hr was followed by a marked slowing, or pause, in growth that lasted for a period of at least 1–2 hr. In this period the turbidity value was usually about 0·3, only a little higher than the value in the anaerobic cultures, and it may therefore be concluded that the check to growth was due to exhaustion of the dissolved oxygen and fermentable substrates in the broth. Thereafter, at some time between 6 and 12 hr, the Fim(1)$^+$ cultures formed a pellicle and entered on a marked and prolonged phase of renewed

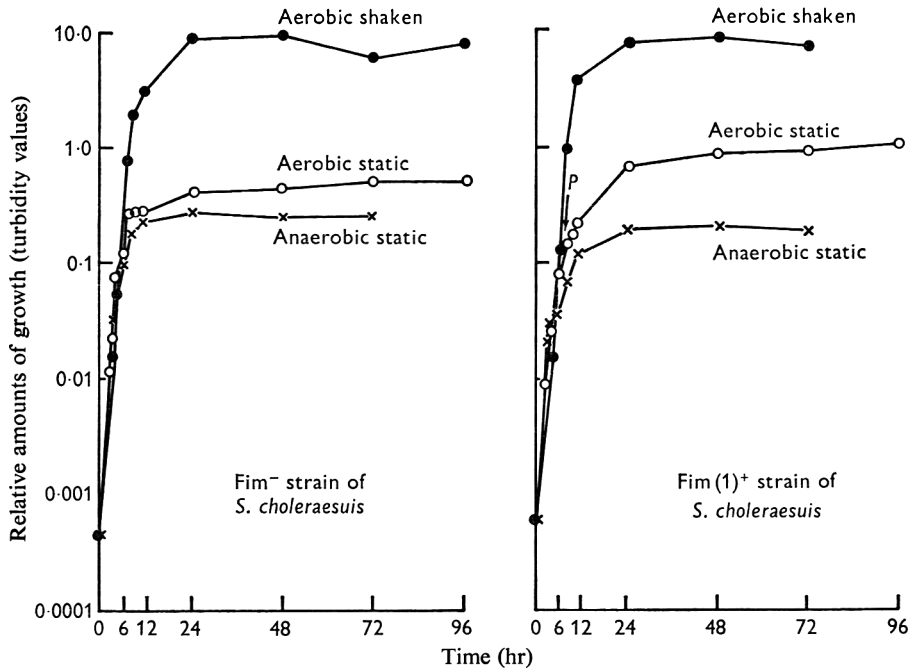

Fig. 1. Amount of growth (log scale) of Fim$^-$ strain NCTC 5364 of *Salmonella choleraesuis* (left) and Fim (1)$^+$ strain NCTC 5738 of *S. choleraesuis* (right) in broth incubated at 37°; aerobic and static, O——O; anaerobic in hydrogen, ×——×; aerobic and shaken, ●——●. The inocula were from broth cultures grown for 48 hr after they had been inoculated from a preceding 48 hr broth culture; that of strain NCTC 5738 was strongly haemagglutinating and in the fimbriate phase. *P* shows the time at which a pellicle first became visible in the aerobic static cultures.

growth. Their turbidity values increased and commonly reached about 1·0 at 24 hr, 1·5 at 48 hr and 2·0 at 96 hr. In contrast, the cultures of most of the Fim$^-$ salmonellas did not form pellicles. They showed much slower and less extensive post-logarithmic growth than the Fim(1)$^+$ cultures, and their turbidity values reached only about 0·4 at 24 hr and about 0·8 at 48–96 hr. Thus, throughout the period 24–96 hr their bacterial concentration was only about half as great as that in the cultures of comparable type-1 fimbriate bacteria.

Pellicle-forming Fim$^-$ Salmonella typhimurium. Motile Fim$^-$ strains of *S. typhimurium* differed from most other Fim$^-$ salmonellas by forming a pellicle in aerobic static broth cultures. The pellicle usually appeared at between 24 and 72 hr, i.e. much later than in Fim(1)$^+$cultures, and its appearance was followed by a phase of renewed growth. The turbidity value in the Fim$^-$ cultures was only about half that in the com-

parable Fim(1)$^+$ cultures during the period 24–48 hr, but after the formation of the pellicle it increased more rapidly and by 96 hr it approached the value in the Fim(1)$^+$ cultures (Fig. 2).

Non-fimbriate-phase inocula. Most Fim(1)$^+$ salmonellas rapidly become converted into the fimbriate phase when they are cultured in aerobic static broth from an inoculum consisting of agar-grown bacteria most of which are in the non-fimbriate phase. They are thus able to form a pellicle as early as 8–12 hr and then begin a phase of secondary growth. This behaviour is exemplified in the growth curve of the Fim(1)$^+$ strain of *Salmonella typhimurium* shown in Fig. 2. The Fim(1)$^+$ inocula in this experiment were taken from a suspension made from a single colony on a 24 hr agar culture

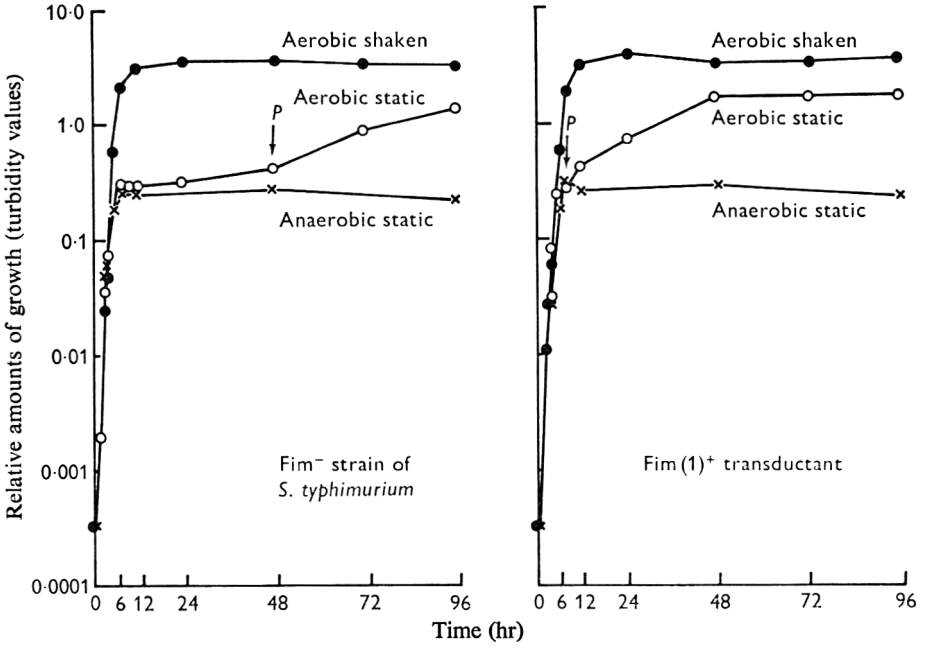

Fig. 2. Amount of growth of Fim$^-$ strain S575 of *S. typhimurium* (left) and its Fim (1)$^+$ transductant, S575F, (right) in broth incubated at 37°; aerobic and static, O——O; anaerobic in hydrogen, ×——×; aerobic and shaken, ●——●. The inocula were from a single colony of each strain grown for 24 hr on agar; the inoculated bacteria of the Fim (1)$^+$ strain were mainly in the non-fimbriate phase. *P* shows the time at which a pellicle first became visible in the aerobic static cultures.

that showed only weak haemagglutinating activity. Nevertheless, the aerobic static cultures grown from these inocula formed a pellicle at about 8 hr and became strongly haemagglutinating within 48 hr. The anaerobic static and aerobic shaken cultures grown from the same inocula remained mainly in the non-fimbriate phase and were still only weakly haemagglutinating after 48 and 96 hr.

A few Fim(1)$^+$ salmonellas are slower than the majority in changing into the fimbriate phase when cultured in aerobic static broth. Strain NCTC 5738 of *Salmonella choleraesuis* is outstanding in this respect. When subcultured serially on agar it gives growths that are entirely non-fimbriate and non-haemagglutinating, and when, from a non-fimbriate inoculum, it is subcultured either serially or for a long period in aerobic static broth, it becomes richly fimbriate and strongly haemagglutinating. The inocula

of this strain that were used in the experiment shown in Fig. 1 were taken from a fimbriate-phase broth culture; for this reason a pellicle was formed and secondary growth started at an early stage (8 hr) in the aerobic static cultures. In a parallel experiment, which is not illustrated, the inocula consisted of non-fimbriate-phase bacteria from a colony grown on agar for 24 hr. The aerobic static cultures grown from these inocula became detectably fimbriate and formed a pellicle only after about 48 hr. Their post-logarithmic growth was slow and delayed as compared with that in cultures inoculated with fimbriate-phase bacteria, but eventually, in 96 hr, they attained a bacterial concentration as high as that in the fimbriate-inoculated cultures.

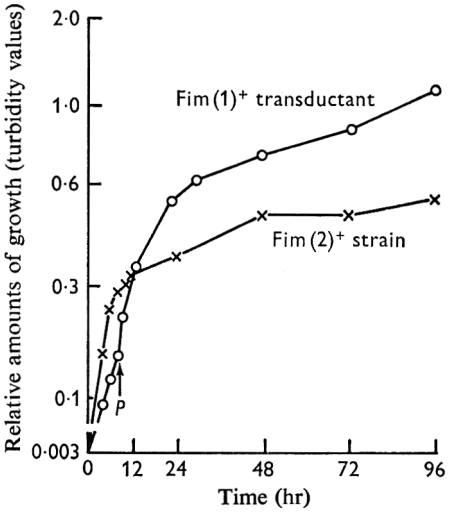

Fig. 3. Amount of growth of the non-haemagglutinating Fim(2)+ strain NCTC 5707 of *S. paratyphi B*, ×——×, and that of its haemagglutinating Fim(1)+ transductant NCTC 5707 HA+, O——O, in phosphate-buffered broth incubated aerobically and statically at 37°. The inocula were from fimbriate-phase broth cultures grown for 24 hr after inoculation from 48 hr broth cultures. *P* shows the time at which a pellicle first became visible in the cultures of the Fim(1)+ cultures. A pellicle was not formed in the Fim(2)+ cultures.

Fim(2)+ bacteria. The importance of the haemagglutinating property of the fimbriae for pellicle formation and secondary growth was demonstrated in an experiment comparing the growth curve of an organism bearing haemagglutinating (type-1) fimbriae with that of a related organism bearing non-haemagglutinating (type-2) fimbriae. The Fim(2)+ organism was strain NCTC 5707 of *Salmonella paratyphi B* and the Fim(1)+ organism was a haemagglutinating variant derived from NCTC 5707 by phage transduction. Aerobic static broth cultures were grown from fimbriate-phase inocula taken from a 24 hr broth culture, and their growth curves are shown in Fig. 3. The parent strain with type-2 fimbriae behaved like a non-fimbriate organism: it neither formed a pellicle nor gave a marked post-logarithmic phase of growth, and it attained only a moderate bacterial concentration (turbidity 0·55) after 96 hr. In contrast, the transductant with type-1 fimbriae formed a pellicle at 8 hr, gave a large secondary phase of growth and attained a final bacterial concentration that was more than twice as great (turbidity 1·3).

Effect of D-mannose and α-methylmannoside on pellicle formation and growth

Pellicle formation and secondary growth by Fim(1)$^+$ organisms in aerobic static broth was prevented or much delayed by the addition to the broth of either D-mannose or α-methylmannoside (methyl-α-D-mannose), substances exceptional among sugars in having a powerful inhibitory effect on the haemagglutinating activity of Fim(1)$^+$ bacteria (Duguid & Gillies, 1957). Cultures of the Fim(1)$^+$ strain LT2 of *Salmonella typhimurium* were grown under aerobic static conditions at 37° in tubes of 10 ml. broth containing 0·2% (w/v) of one of these haemagglutination-inhibiting sugars. Control cultures were grown in broths containing 0·2% (w/v) of one of the non-haemagglutination-inhibiting sugars, D-glucose and L-sorbose, and in broth without added sugar. The broth was buffered at pH 7·0 by the addition of phosphate (KH_2PO_4, 0·35 g, Na_2HPO_4, 0·65 g., per 100 ml.) because Duguid *et al.* (1966) had found that salmonella cultures grown in unbuffered broth containing 1% (w/v) glucose became highly acid (pH 4·2–5·0) and that the acid reaction inhibited the development of fimbriae. In our cultures of strain LT2 grown in phosphate-buffered broth containing 0·2% glucose the pH value did not fall below 6·0 and fimbriae were well developed. The tubes of broth were each inoculated with about 3×10^7 bacteria from a 24 hr fimbriate-phase culture grown in sugar-free broth; this latter broth had itself been inoculated from a 48 hr broth subculture from a colony on agar.

Table 3 shows the results of an experiment with the wild-type strain LT2, an organism that ferments and utilizes D-glucose and D-mannose, but not L-sorbose or α-methylmannoside. The results for the cultures in the broths containing the non-haemagglutination-inhibiting sugars, glucose and sorbose, were similar to those for the cultures in the broth without added sugar. Pellicles were formed in all three media in about 6 hr and an extensive phase of secondary growth took place during the subsequent 24 hr. Strong haemagglutinating activity, indicating rich fimbriation, was present after 12 hr. In the first 8 hr, growth was rather faster in the glucose broth than in the sorbose and plain broths, probably because the glucose was utilized for the production of energy.

Cultures with mannose. The course of growth in mannose broth was similar to that in glucose broth, except that pellicle formation was delayed by about 2–3 hr and post-logarithmic growth was rather slower. The fact that pellicle formation was not delayed to a much later time than 8 hr is explained by the finding that the mannose was rapidly utilized and removed from the medium. Each culture was tested for residual mannose by examining a drop of centrifuged culture supernatant fluid for the ability to inhibit haemagglutination when added to a drop of red blood cells mixed with fimbriate bacteria. Inhibitory activity was not detected in the cultures that had been grown for 12 hr or longer, and it is concluded that the mannose content of these cultures had been decreased to less than 0·001% (w/v), which is the minimum haemagglutination-inhibiting concentration. The reason why a pellicle was formed at 8 hr, about 4 hr before the time of exhaustion of the mannose from the broth as a whole, may have been that mannose was exhausted earlier from the surface layer of the broth where bacteria were concentrated by aerotaxis.

Cultures with α-methylmannoside. A much more prolonged inhibition of pellicle formation was observed with α-methylmannoside. This sugar was not utilized by strain LT2, and haemagglutination-inhibitory activity was still detected in cultures

Table 3. *The influence of haemagglutination-inhibiting and -non-inhibiting sugars on pellicle formation and the amount of growth of the fimbriate strain LT2 of Salmonella typhimurium in phosphate-buffered broth*

Time of incubation (hr)	Results for culture in buffered broth with the addition of (0·2%, w/v) Nil: Amount of growth*	Nil: Presence of pellicle	Sorbose: Amount of growth	Sorbose: Presence of pellicle	Glucose: Amount of growth	Glucose: Presence of pellicle	Mannose: Amount of growth	Mannose: Presence of pellicle	Mannose: HA-inhibiting activity in broth†	α-Methylmannoside: Amount of growth	α-Methylmannoside: Presence of pellicle	α-Methylmannoside: HA-inhibiting activity in broth†
0	(0·003)	−	(0·003)	−	(0·003)	−	(0·003)	−	+	(0·003)	−	+
2	0·02	−	0·01	−	0·02	−	0·01	−	+	0·02	−	+
4	0·08	−	0·07	−	0·12	−	0·12	−	+	0·10	−	+
6	0·21	+	0·18	+	0·85	+	0·55	−	+	0·23	−	+
8	0·29	+	0·26	+	1·04	+	0·75	+	+	0·25	−	+
10	0·65	+	0·36	+	1·13	+	0·80	+	+	0·27	−	+
12	0·84	+	0·75	+	1·19	+	0·92	+	−	0·26	−	+
14	0·92	+	0·84	+	1·32	+	1·06	+	−	0·28	−	+
24	1·55	+	1·31	+	1·62	+	1·32	+	−	0·34	−	+
27	1·51	+	1·45	+	1·62	+	0·91	+	−	0·34	−	+
30	1·62	+	1·40	+	1·96	+	1·05	+	−	0·38	−	+
48	2·13	+	1·78	+	2·33	+	1·62	+	−	1·19	+	+
51	2·11	+	1·86	+	2·39	+	2·14	+	−	1·00	+	+
72	2·25	+	1·95	+	2·78	+	2·84	+	−	1·49	+	+
96	2·16	+	1·98	+	2·16	+	2·78	+	−	1·49	+	+
120	2·60	+	2·36	+	2·99	+	3·10	+	−	1·94	+	+
144	2·40	+	2·32	+	2·94	+	3·03	+	−	1·85	+	+

* The amount of growth is given as the turbidity value obtained with the Spekker absorptiometer. The result for 0 hr is the turbidity equivalent of the concentration of the bacteria that were inoculated into the 10 ml. volume of medium.

† +, denotes the presence of unutilised mannose or α-methylmannoside in the broth; −, denotes the absence of haemagglutination-inhibitory concentrations of mannose or α-methylmannoside in the broth, i.e. utilization of the sugar is complete.

that had been incubated for 6 days. A pellicle did not appear in any of these cultures during the first 30 hr, so that pellicle formation was delayed by at least 24 hr beyond the time of its occurrence in the control cultures (6 hr). A pellicle did appear after 48 hr, despite the continuing presence of the α-methylmannoside. This late formation of a pellicle was probably due, not to an eventual failure of inhibition of the pellicle-forming action of the fimbriae, but to the kind of process that is responsible for the formation of a pellicle by non-fimbriate strains of *Salmonella typhimurium* when incubation is continued for as long as 48 hr.

The delay in pellicle formation until later than 30 hr in the cultures with α-methylmannoside was associated with a corresponding delay in the onset of the phase of secondary growth. Thus, the turbidity value increased only slightly between 6 hr, when it was 0·23, and 30 hr, when it was 0·38. This increase of less than twofold is to be compared with the eight-fold increase, from 0·21 at 6 hr to 1·62 at 30 hr, in the control cultures without added sugar. Since growth during the first 6 hr of incubation was just as rapid in the cultures with α-methylmannoside as in those without it, it may be concluded that α-methylmannoside was not toxic to the bacteria, or inhibitory to growth in any way other than through its effect on pellicle formation.

Haemagglutination tests made with bacteria separated from the cultures by centrifugation and washed twice in saline to free them from the sugar showed that the cultures were poorly fimbriated during the first 24 hr of growth and moderately richly fimbriated after 48–96 hr. It is seen, therefore, that the effect of the α-methylmannoside was to inhibit the pellicle-forming activity of the fimbriate bacteria, and not to inhibit the development of fimbriae. The degree of fimbriation was certainly rather poorer in the cultures with α-methylmannoside than in the control cultures, and the reason for this difference was probably the absence of selective growth of fimbriate-phase bacteria in an early forming pellicle.

Non-mannose-fermenting mutants

A clear demonstration of the pellicle-inhibiting effect of D-mannose itself was obtained in experiments with two non-mannose-fermenting mutant strains, SL1031M and SL970. The growth curves of strain SL1031M in plain broth and broths with glucose, sorbose, mannose and α-methylmannoside are shown in Fig. 4. Like its parent strain, LT2, strain SL1031M formed a pellicle at 6 hr when grown in plain broth, glucose broth or sorbose broth. Glucose was not fermented by this mutant during the first 30 hr of culture, and cultures in all three media showed a similar secondary phase of growth during the 24 hr following pellicle formation. After 30 hr the utilization of glucose led to greater amounts of growth being obtained in the glucose broth than in the other media.

Pellicle formation by strain SL1031M was delayed until after 30 hr both in the broth with mannose and in that with α-methylmannoside. As can be seen in Fig. 4, the absence of early pellicle formation in these media was associated with the absence of a phase of secondary growth in the period 6–30 hr. In this period the bacterial concentrations were much less than those in the plain, glucose and sorbose broths. When pellicles were eventually formed at about 48 hr in the mannose and α-methylmannoside broths, a late secondary phase of growth took place. Tests for the presence of haemagglutination-inhibitory activity in supernatant fluids of the cultures showed that α-methylmannoside was still present after 96 hr and that mannose was still present after

48 hr. By 72 hr, all the mannose had disappeared, apparently as a result of the emergence of mannose-fermenting back-mutants.

Mutant SL970. Experimentswith the other non-mannose-fermenting strain, SL970, gave generally similar results. The presence of mannose prevented the formation of a pellicle and the onset of secondary growth during the first 72 hr of culture. Bacteria centrifuged from the non-pelliculate 72 hr culture and washed free from residual mannose had moderately strong haemagglutinating activity. The early formation of a pellicle by this strain was not prevented by the addition to broth of 0·2% (w/v) of arabinose, galactose, glucose, lactose, maltose or xylose.

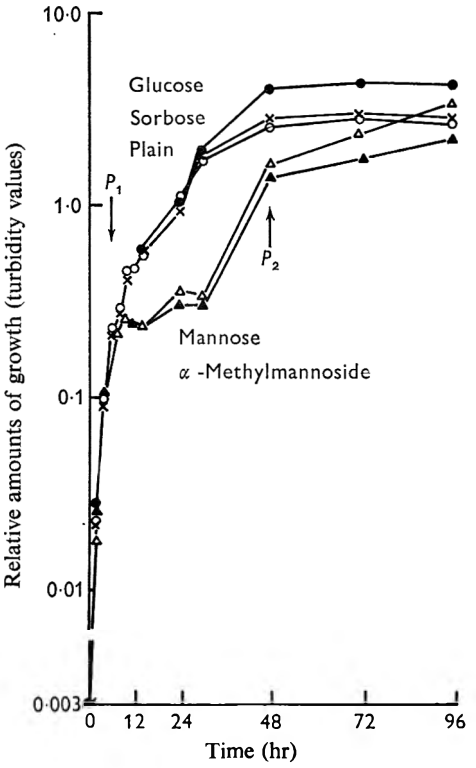

Fig. 4. Amount of growth of non-mannose-fermenting strain SL1031M of *S. typhimurium* grown under aerobic static conditions at 37° in phosphate-buffered broth, O——O, and phosphate-buffered broth containing 0·2% (w/v) of L-sorbose, ×——×, D-glucose, ●——●, D-mannose, △——△, and α-methylmannoside, ▲——▲. The inocula were from a fimbriate-phase broth culture grown for 24 hr after inoculation from a 48 hr broth culture. P_1 shows the time when a pellicle was first seen in the plain, sorbose and glucose broths; P_2 shows that in the mannose and α-methylmannoside broths.

DISCUSSION

Our experiments show that the presence of haemagglutinating type-1 fimbriae in motile salmonellas has a similar effect in promoting rapid surface pellicle formation and marked post-logarithmic growth in aerobic static broth as it has in non-motile shigellas (Duguid & Wilkinson, 1961). The role of type-1 fimbriae in promoting pellicle formation at an early stage, i.e. within the first 12 hr, of culture is indicated by the

finding that early pellicle formation took place in all fimbriate-phase cultures of Fim(1)$^+$ organisms, but not in any culture of a Fim(2)$^+$ or Fim$^-$ organism. The formation of a pellicle at a later stage, i.e. at between 18 and 96 hr, by most cultures of Fim$^-$ strains of *Salmonella typhimurium* must be due to a different and non-fimbrial property that is present in Fim$^-$ *S. typhimurium* but not in other Fim$^-$ salmonellas, which do not form a pellicle at any stage.

The fimbrial pellicle usually appeared 2–4 hr earlier in the cultures of motile Fim(1)$^+$ salmonellas than in those of non-motile Fim(1)$^+$ shigellas. Motility, therefore, seems to accelerate, rather than hinder pellicle formation. Possibly the acceleration is the result of the motile fimbriate bacteria gathering rapidly at the surface of the broth by aerotactic migration. Non-pellicle-forming cultures of highly motile Fim$^-$ salmonellas did not give marked post-logarithmic growth; e.g. non-pellicle-forming cultures of the motile Fim$^-$ strain SL272 of *Salmonella typhimurium* did not give significantly greater amounts of growth in 24–96 hr than non-pellicle-forming cultures of this strain's non-motile variants, SW573 and SW578. Apparently, the aerotactic migration of bacteria towards the broth surface is not, by itself, nearly as effective a means of procuring the access of large numbers of bacteria to atmospheric oxygen as is the growth of the bacteria in a surface pellicle.

The finding that the growth of non-pellicle-forming cultures in aerobic static broth was checked, at about 6 hr, at a bacterial concentration only a little higher than in anaerobic cultures, and then increased only slowly and slightly during further incubation up to 96 hr, suggests that the limitation of growth in these cultures was due to the exhaustion of substrates capable of yielding energy by fermentation and that the production of energy by oxidation of non-fermentable substrates was severely limited by the small amount of oxygen dissolved in the broth. This explanation is supported by the observation that primary growth proceeded unchecked to a tenfold higher value in the cultures that were efficiently aerated by continuous shaking.

Pellicle formation in aerobic static cultures was associated with a subsequent phase of renewed growth great enough to raise the bacterial concentration to a value approaching that in the aerobic shaken cultures. This marked post-logarithmic growth took place in the period 24–48 hr after the formation of the pellicle; it did not occur in the non-pellicle-forming cultures. The association of post-logarithmic growth with pellicle formation, and the finding that relatively little post-logarithmic growth took place in the aerobic static cultures of fimbriate bacteria in which pellicle formation was prevented by the addition of mannose or α-methylmannoside, are strong evidence that pellicle formation was responsible for the renewal of growth in the post-logarithmic period. The effect of the pellicle is presumably due to its numerous contained bacteria having free use of atmospheric oxygen for the production of energy from non-fermentable substrates in the broth. The absence of pellicle formation and post-logarithmic growth in anaerobically incubated cultures agrees with this explanation.

Two observations suggest that the property of type-1 fimbriae which confers their mannose-sensitive adhesiveness and haemagglutinating activity is also the property which promotes pellicle formation. One is that salmonella organisms with non-haemagglutinating type-2 fimbriae do not form a pellicle. The other is that the addition to broth of mannose or α-methylmannoside prevents or delays the formation of a pellicle by organisms with haemagglutinating type-1 fimbriae. Since, out of the series of sugars tested, only mannose and α-methylmannoside inhibited pellicle formation

by Fim(1)$^+$ bacteria and since only these two sugars inhibit haemagglutination by such bacteria (Duguid & Gillies, 1957), it seems that the same specific mechanism is involved in the inhibition of pellicle formation as in the inhibition of haemagglutination. The nature of this mechanism is unknown. Duguid & Gillies found that mannose did not become firmly bound to, or produce an irreversible change in, either the fimbriate bacteria or the red blood cells. When the mannose was removed from the bacteria or from the red cells, by centrifugation and washing, the bacteria regained strong haemagglutinating activity and the red cells regained full agglutinability. Because of this reversibility of the effect, it could not be determined whether the action of the mannose was primarily on the bacteria or on the red cells. Our finding that mannose inhibits pellicle formation by fimbriate bacteria shows that the sugar does have some direct action on the bacteria. Possibly it absorbs reversibly to specific receptor sites on the type-1 fimbriae and thereby alters the surface properties of the fimbriae so that they cannot adhere to red cells or promote pellicle formation. How the mannose-sensitive haemagglutinating factor brings about pellicle formation is unknown. Conceivably it does this either by rendering the bacteria hydrophobic and liable to float on the surface of the broth or by causing bacteria already on the surface of the broth to adhere to one another.

There is no clear evidence that fimbriae of types other than type 1 have the property of promoting pellicle formation. Fim(3)$^+$ strains of *Klebsiella aerogenes*, i.e. strains bearing only type-3 fimbriae with mannose-resistant adhesive properties, have been found to form pellicles after culture for 12–72 hr (Duguid, 1959). Type-3 fimbriae do not occur in salmonellas (Duguid *et al.* 1966). Type-F fimbriae (F pili) (Crawford & Gesteland, 1964; Brinton, Gemski &Carnahan, 1964), which are thought to be organs of bacterial conjugation, were not seen with the electron microscope in any of our Fim$^-$ salmonellae. Since we did not use F-specific phage, we would not have recognized the presence of these scanty F fimbriae in our Fim(1)$^+$ and Fim(2)$^+$ organisms; but it seems unlikely that any were present because factor F is rarely found in salmonellas.

Our results agree with the possibility that saprophytic bacteria which live in stagnant, poorly oxygenated waters containing oxidizable nutrients may benefit from the function of fimbriae in promoting pellicle formation and access to atmospheric oxygen. In the laboratory, the effect of fimbriae in promoting pellicle formation and post-logarithmic growth is important in two experimental procedures. It is probably responsible for the success of the methods of prolonged and serial broth culture in obtaining selective outgrowth of fimbrate-phase bacteria from a non-fimbriate-phase inoculum (Duguid & Gillies, 1957). It is probably also responsible for the success of these methods in obtaining the selective outgrowth of rare Fim(1)$^+$ transductant bacteria from mixtures of Fim$^-$ bacteria with phage propagated on Fim(1)$^+$ donor bacteria. Indeed, in observations made with mixed cultures of distinctively marked Fim(1)$^+$ and Fim$^-$ strains of *Salmonella typhimurium* we have obtained direct proof that the conditions of culture in aerobic static broth are highly selective for fimbriate bacteria. The cultures were grown from mixed inocula consisting of a small number (about 10) of bacteria of the rhamnose-fermenting Fim(1)$^+$ strain S206 and a large number (about 10^8) of bacteria of the non-rhamnose-fermenting Fim$^-$ strain M7471. After aerobic static incubation for 48 hr, the Fim(1)$^+$ bacteria comprised 10–20% of the total bacterial population; the extent of their multiplication was about one million times greater than that of the Fim$^-$ bacteria. They showed little or no outgrowth in mixed cultures grown

under anaerobic static or aerobic shaken conditions. We attribute this strong selective advantage of Fim(1)$^+$ over Fim$^-$ bacteria in aerobic static broth cultures to the pellicle-forming property of the fimbriae.

We wish to thank Dr E. S. Anderson and Dr B. A. D. Stocker for providing us with strains and information about their characters. This work was supported in part by a grant to one of us (J.P.D.) from the Fleming Memorial Fund.

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J. gen. Microbiol. (1968), **51**, 17–22
With 1 *plate*
Printed in Great Britain

Inhibition of Excystment of *Schizopyrenus russelli* Cysts in the Presence of Emetine and its Cysticidal Effect in Conjunction with Sodium Lauryl Sulphate

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(*Accepted for publication* 31 *August* 1967)

SUMMARY

Excystment agents (extract of *Escherichia coli*, living *E. coli* and glutamic acid) failed to cause excystment of *Schizopyrenus russelli* cysts in the presence of either emetine or compounds I and II (structurally based on emetine). A very high percentage of the cysts excysted in the presence of the excystment agents after the removal of emetine, showing that the treated cysts were viable. Ninhydrin reaction, using a fixed amount of glutamic acid and increasing concentrations of emetine, showed progressive inhibition of colour development. This suggests the possible binding of excystment factor to emetine, thus preventing excystment. Excystment inducing property of the excystment agents could not be prevented in the presence of carbarsone. When the cysts were treated with sodium lauryl sulphate and then with emetine or with sodium lauryl sulphate and emetine together, there was hardly any excystment. Sodium lauryl sulphate rendered the cyst wall permeable to emetine and the latter killed the cysts.

INTRODUCTION

Relapses encountered commonly in the treated cases of human intestinal amoebiasis seem to be due to the *in vivo* persistence of cysts of *Entamoeba histolytica* which escape the action of drugs. Known amoebicides appear to have little or no effect on the cystic stage of *E. histolytica*. 5 % emetine HCl and yatren were found to have no effect on the cysts of *E. histolytica* treated for 30 min. *in vitro* (Yorke & Adams, 1926). It is, therefore, important to discover drugs that are, in addition to having amoebicidal property, cysticidal or prevent amoebae from forming cysts or make the amoebae come out of the cysts. This question has not, so far, seriously attracted the attention of biologists and chemists engaged on the chemotherapy of intestinal amoebiasis.

Excystment of cysts of free-living amoebae and *Entamoeba histolytica* in the presence of certain living bacteria has been noted by several workers (Crump, 1950; Drozanski, 1961; Dudziak, 1955; Kunicki-Goldfinger *et al.* 1957; Singh, and his co-workers, 1956, 1958, 1963, 1965.). Singh, Mathew & Anand (1958) and Singh, Saxena & Iyer (1965) found that an aqueous extract of an *Aerobacter* sp. and *Escherichia coli* caused excystment of five species of free-living amoebae. It was further shown by them that certain amino acids present in the aqueous extract were responsible for the excystment of *Schizopyrenus russelli* cysts. Certain chemically pure amino acids, at suitable pH range, were also found to cause excystment of

S. russelli cysts. These observations have been confirmed by Drozanski (1961) in the case of free-living amoebae.

In this communication the work carried out on the inhibition of excystment of *Schizopyrenus russelli* cysts in the presence of emetine and its cysticidal effect in conjunction with an anionic surfactant sodium lauryl sulphate is presented.

METHODS

Cysts from a 'pure line' culture of *Schizopyrenus russelli* were used in this work. The amoebae were grown on non-nutrient agar (2·5 % (w/v) agar, 0·5 % (w/v) NaCl; pH 6·8–7·0) plates supplied with a young culture (3 days old) of *Escherichia coli*, grown on nutrient agar slopes, as food. Three- to seven-day-old cysts were harvested and viable sterile cysts, free from living and dead bacteria, were obtained by the method of Singh *et al.* (1965). For excystment experiments, living *E. coli* cells, autoclaved aqueous extract of *E. coli* or L-glutamic acid (2 % (w/v), pH 6·0) were used either singly or with emetine, compounds I and II, or carbarsone. Fifty to seventy-five cysts were put as a hanging drop suspension in a cavity slide ($25° \pm 1°$) and % excystment was calculated from the count of the amoebae and the unexcysted cysts. It may be pointed out that *S. russelli* forms a double-walled cyst and excystment takes place in two stages. First, the inner wall disappears and a small amoeba moves freely within the outer wall. In a successful case, the outer wall gives way and the amoeba emerges (Pl. 1, fig. 1–3). A cyst was considered excysted only when an amoeba escaped from it and was found moving freely in the surrounding medium.

Free amino acid available for excystment in mixtures of amino acid (glutamic acid) containing different concentrations (w/v) of emetine-HCl (62·5–1000 μg./ml.) or compounds I and II (1000 μg./ml.) or carbarsone (1000 μg./ml.) was observed by ninhydrin reaction. Emetine-HCl and carbarsone used were from Burroughs Wellcome and Co., and compounds I (4-(β-phenylethylaminomethyl)1-phenylethyl piperidine) and II (4-(6,7-dimethoxy 1,2,3,4 tetrahydro-1-isoquinoliomethyl) 1-β-3,4-dimethoxy-phenylethyl piperidine) were synthesized in the Chemistry Division of this Institute as potential amoebicidal agents based on the structure of emetine.

Cysts were treated with different concentrations of sodium lauryl sulphate (made in water or M/15 phosphate buffer, pH 7·0) followed by emetine or together with emetine. Cysts treated individually with emetine or sodium lauryl sulphate were always included as controls. The treated cysts were washed with double glass distilled water, and the % excystment was determined.

RESULTS

The data presented in Table 1 clearly show that aqueous extract of *Escherichia coli*, live *E. coli* and glutamic acid failed to cause excystment of *Schizopyrenus russelli* cysts in the presence of emetine. With *E. coli* extract, excystment was observed to the extent of 89 %, whereas in the presence of emetine-HCl (126 μg./ml.) the % excystment was only 6. When the emetine concentration was 1000 μg./ml., there was no excystment. These cysts did not show any excystment in the presence of emetine on prolonged incubation for 48, 72, 96 and 144 hr. Morphologically the treated cysts seemed to be normal (Pl. 1, fig. 4). In 62.5 μg./ml. emetine the cysts were found to excyst in high proportion. It may be mentioned that addition of emetine-HCl did not appreciably

alter the pH of the bacterial extract from its original value of 6·5. Once emetine was washed off, a very high percentage of the cysts excysted in the presence of *E. coli* extract (Table 1).

Living *Escherichia coli* cells also failed to cause excystment in the presence of emetine-HCl (1000 μg./ml.). The cysts appeared normal (Pl. 1, fig. 5) and nearly all the cysts excysted readily in the presence of living *E. coli* when emetine was removed.

Table 1. *Inability of excystment inducing agents to cause excystment of Schizopyrenus russelli cysts in the presence of emetine**

Cysts treated with	No. of cysts	No. of cysts excysted	% excystment
E. coli extract	166	148	89
Emetine (1000 μg./ml.); excystment with *E. coli* extract after removal of emetine	183	180	98
E. coli extract + emetime (1000 μg./ml.)	443	Nil	Nil
Excystment with *E. coli* extract after the removal of emetine	451	410	91
E. coli extract + emetine (125 μg./ml.)	254	14	6
Excystment with *E. coli extract after the* removal of emetine	157	140	89
Live *E. coli*	300	294	98
Live *E. coli* + emetine (1000 μg./ml.)	599	Nil	Nil
Excystment with live *E. coli* after the removal of emetine	494	494	100
Glutamic acid (2 %, pH 6·0)	150	128	85
Glutamic acid (2 %, pH 6·0) + emetine (1000 μg./ml.)	590	Nil	Nil
Excystment with glutamic acid after the removal of emetine	418	384	92

* The cysts were treated for 24 hr with emetine alone or with emetine and excystment agents together.

Glutamic acid gave an excystment of 85 %, whereas glutamic acid containing emetine-HCl (1000 μg./ml.) showed no excystment during a period of 72 hr. After the removal of emetine, these cysts could excyst normally (92 %) with fresh glutamic acid (Table 1). Lower concentration of emetine-HCl (125 μg./ml.) in glutamic acid, however, did not inhibit excystment but only delayed the excystment process. Nearly all the cysts excysted within 48 hr. Still lower concentration of emetine-HCl (62·5 μg./ml.) did not affect the excystment in the presence of glutamic acid.

Ninhydrin reaction was run in a parallel experiment and a progressive inhibition of colour development was noticed when increasing amount of emetine were added to a fixed amount of glutamic acid. When this *in vitro* system was run chromatographically (butanol + acetic acid + water: 4 + 1 + 5) emetine and glutamic acid could be completely separated.

Compounds I and II (1000 μg./ml.) also completely inhibited the excystment of *Schizopyrenus russelli* in the presence of *Escherichia coli* extract up to 72 hr. When they were removed, nearly all the cysts excysted in the presence of *E. coli* extract. In this respect the action of these compounds was like that of emetine-HCl, although they have

not proved to be amoebicidal. It may be mentioned that carbarsone (1000 μg./ml.) did not inhibit but only delayed excystment in the presence of *E. coli* extract.

Cysts treated with sodium lauryl sulphate at dilutions of 500, 250 and 125 μg./ml. (in M/15 phosphate buffer, pH 7·0) for 1 hr, and washed in double glass-distilled water, gave 74, 79 and 76 % excystment respectively in the presence of aqueous extract of *Escherichia coli* (Table 2). The % excystment of the untreated cysts was 95 (Table 2). When the cysts were treated with emetine (1000 μg./ml.) for 24 hr. after sodium lauryl sulphate (500 μg./ml.) treatment, and washed in distilled water, there was hardly any excystment (Table 2).

Table 2 *Lethal effect of emetine on cysts of Schizopyrenus russelli treated with sodium lauryl sulphate followed by emetine*

Treatment	% excystment after sodium lauryl sulphate treatment for 1 hr			% excystment after sodium lauryl sulphate treatment for 1 hr followed by emetine (1000 μg./ml.) for 24 hr		
	No. of cysts	No. of cysts excysted	% excystment	No. of cysts	No. of cysts excysted	% excystment
Sodium lauryl sulphate (500 μg./ml.)	334	247	74	142	1	1
Sodium lauryl sulphate (250 μg./ml.)	213	168	79	157	39	25
Sodium lauryl sulphate (125 μg./ml.)	489	373	76	322	213	66
E. coli. extract (control)	538	511	95	—	—	—

Table 3. *Lethal effect of emetine on cysts of Schizopyrenus russelli when treated with sodium lauryl sulphate and emetine together*

Treatment	Sodium lauryl sulphate in phosphate buffer. % excystment after 48 hr.			Sodium lauryl sulphate in water. % excystment after 48 hr.		
	No. of cysts	No. of cysts excysted	% excystment	No. of cysts	No. of cysts excysted	% excystment
Sodium lauryl sulphate (62·5 μg./ml.)	333	327	98	149	146	98
Sodium lauryl sulphate + emetine (1000 μg./ml.)	457	4	1	225	6	3
Sodium lauryl sulphate (31·3 μg./ml.)	478	473	99	219	212	97
Sodium lauryl sulphate + emetine (1000 μg./ml.)	609	5	1	510	5	1
Sodium lauryl sulphate (15·6 μg./ml.)	502	499	99	118	116	98
Sodium lauryl sulphate + emetine (1000 μg./ml.)	560	21	4	192	3	2
E. coli extract (control)	144	144	100	189	187	99

In another set of experiments cysts were treated with sodium lauryl sulphate at dilution of 62·5, 31·2 and 15·6 μg./ml. (in M/15 phosphate buffer, pH 7·0, or in distilled water) together with emetine (1000 μg./ml.) for 48 hr. The cysts were then washed and the degree of excystment determined. There was hardly any excystment (Table 3). The % excystment of the cysts treated with sodium lauryl sulphate alone was similar to that of untreated cysts. The cysts appeared normal (Pl. 1, fig. 7) and nearly all the cysts excysted (Table 3).

The cysts which failed to excyst after sodium lauryl sulphate and emetine treatments stained readily in 0·125 % (w/v) aqueous solution of eosin. The protoplasm of these cysts along with the inner cyst wall was very much shrunken (Pl. 1, fig. 6).

DISCUSSION

It has been shown in the present investigation that excystment-inducing agents failed to cause excystment of *Schizopyrenus russelli* cysts in the presence of emetine hydrochloride and compounds I and II. The results of the ninhydrin reaction suggest a possible binding of excystment agent to emetine, whereas, by paper chromatography, the excystment agent and emetine could easily be separated. This binding may thus be mediated by weak electrostatic and van der Waal's forces, as suggested by Dhar (1959). When the cysts are treated with sodium lauryl sulphate and then with emetine or with sodium lauryl sulphate and emetine together, there is practically no excystment. This suggests that sodium lauryl sulphate renders the cyst wall permeable to emetine, the latter killing the cysts. The action of surfactant is not interfered with by the presence of emetine. These findings may have application in eliminating the cysts of *Entamoeba histolytica* from human carrier cases.

It is a great pleasure to express our sincere thanks to Dr M. L. Dhar, Director, Central Drug Research Institute, for his keen interest in this work. Grateful thanks are also due to Dr B. N. Singh, Scientist-in-Charge, Microbiology Division, for helpful suggestions during the course of this work and in the preparation of the manuscript. Authors are thankful to Mr L. M. P. Singh for his technical assistance throughout this investigation.

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EXPLANATION OF PLATE

All the photographs were taken at the same magnification.

Fig. 1. Normal cyst of *Schizopyrenus russelli* with double cyst wall.

Fig. 2. Cyst of *S. russelli* undergoing excystment in *E. coli* extract; inner cyst wall has disappeared and motile amoeba is seen inside the outer cyst wall.

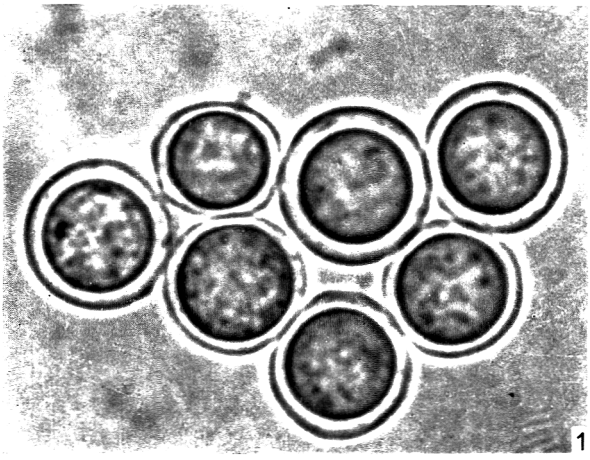
Fig. 3. A motile trophozoite of *S. russelli* after emerging from cyst.

Fig. 4. Cyst of *S. russelli* in *Escherichia coli* extract+emetine (1000 μg./ml.) for 24 hr; appearance normal.

Fig. 5. Cysts of *S. russelli* in the presence of living *E. coli*+emetine (1000 μg./ml.) for 144 hr; appearance normal.

Fig. 6. Cysts of *S. russelli* treated with sodium lauryl sulphate (62·5 μg./ml.) and emetine (1000 μg./ml.) together for 48 hr; the protoplasm along with inner cyst wall is very much shrunken.

Fig. 7. Cyst of *S. russelli* treated with sodium lauryl sulphate (62·5 μg./ml.) for 48 hr; appearance normal.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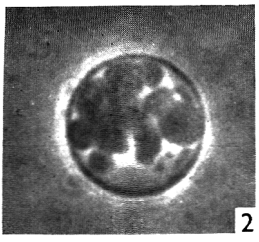
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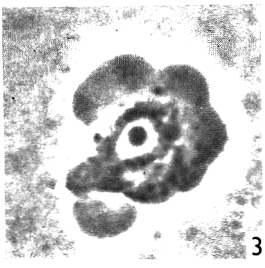
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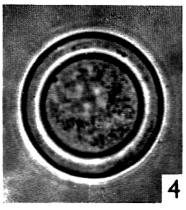
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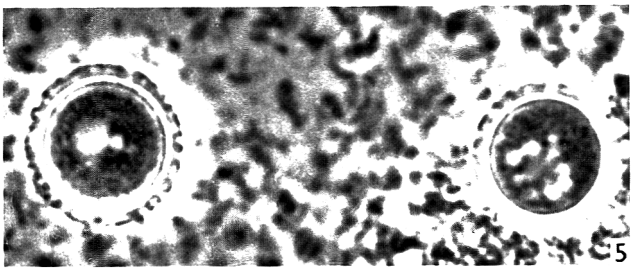
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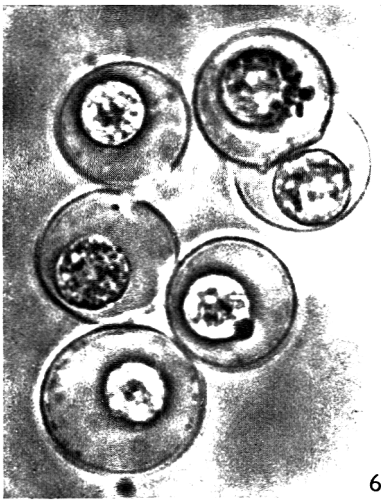
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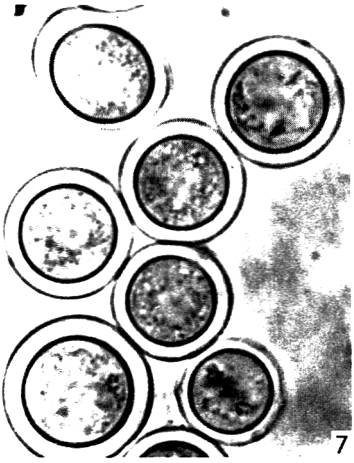
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J. gen. Microbiol. (1968), **51**, 23–33
With 3 plates
Printed in Great Britain

Diversity of Surface Layers in L-forms of *Proteus mirabilis*

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(*Accepted for publication* 1 *September* 1967)

SUMMARY

The structure of surface layers of the bacterial form and different types of the L-form of *Proteus mirabilis* was studied by electron microscopy of thin-sectioned organisms. Morphological data confirm the distinction between the cell wall-less protoplast-L-form and the wall-containing spheroplast-L-form. Organisms of the protoplast-L-form have only one surface-integument, presumably the cytoplasmic membrane. These forms never revert to the bacterial form. The spheroplast-L-form comprises reversible forms (unstable-spheroplast-L-form) as well as non-reverting strains (stable spheroplast-L-form). In both spheroplast-L-forms two surface integuments are always present: a cytoplasmic membrane and a superposed cell wall. In sections of isolated cell walls of normal Proteus bacteria and in wall material spontaneously dissociating from damaged cell walls of spheroplast-L-forms the triple-layered 'unit membrane' is a prominent feature. Thin sections of isolated cell wall lipopolysaccharide identify the unit membrane as a specific structure of this polymer. The thickness of the murein (syn. mucopeptide, mucopolymer) in isolated murein layers ('murein sacculi', Weidel & Pelzer, 1964) from cell walls of normal Proteus bacteria was found to be approximately 20–25 Å.

INTRODUCTION

The task of describing and defining bacterial L-forms has always been a problematic and perplexing one. The organisms are best known for their negative qualities, and their elusive nature is strikingly characterized in their description as: 'The stable growth which consists of soft, protoplasmic elements without defined morphology, which no longer possesses rigid, bacterial forms nor reverts to them...' (Klieneberger-Nobel, 1960). Much remains to be learned about their origin. There is, however, general agreement that the aberrant L-form morphology and the lack of solid cell envelopes is due to functional deficiencies in the biosynthetic apparatus of the bacterial cell wall. The comparative study of surface structures in L-forms and their original bacterial forms is therefore of considerable interest, since it may provide information on the functions and requirements of the morphogenetic apparatus which establishes specific shape and stability in the normal bacterial cell wall. Early work on the penicillin-induced L-forms of *Proteus mirabilis* indicated that the L-form character may be related to very different levels of cell wall damage. These observations are now best understood on the basis of cell-wall models which have been derived from extensive analytical and electron microscopical work on cell walls of *Escherichia coli* and

P. mirabilis. These cell walls are constructed on the foundation of a cell-shaped murein (syn. mucopeptide, mucopolymer) layer ('murein sacculus', Weidel & Pelzer, 1964). Two other macromolecular wall components are also present in large amounts: lipopolysaccharide (LPS), and lipoprotein. These polymers are anchored to the murein sacculus to form an intricate mosaic pattern of functional surface sites (antigens, bacteriophage receptors) whose structural details are still largely unknown (Weidel, 1958; Weidel, Frank & Martin, 1960; Martin & Frank, 1962; Weidel & Pelzer, 1964; Martin, 1964). The stable (= irreversible) L-form of *P. mirabilis* was found to lack both murein components and bacteriophage receptors and thus, like protoplasts of Gram-positive bacteria, seemed to have no cell wall (Kandler & Zehender, 1957; Taubeneck, Böhme & Schumann, 1958; Taubeneck, 1961). In contrast, the unstable (= reversible) L-form contained murein as well as phage receptors (Kandler, Hund & Zehender, 1958; Taubeneck *et al.* 1958). Thin sections of the unstable L-forms revealed the presence of cell walls, and this suggested a similarity of these organisms with the penicillin- or lysozyme-induced spheroplasts of *E. coli* (Hofschneider & Lorek, 1962). More recently a third variety of Proteus L-forms has been described (Martin, 1964). This type is non-reverting, i.e. stable. It does, however, retain a cell wall, including a fragile, balloon-shaped murein sacculus. Proteus L-forms may thus be classified into three types according to the presence or absence of cell walls and the ability or failure to revert to the bacterial form upon transfer to penicillin-free medium:

(1) A stable protoplast-L-form which is not able to revert; cell walls are absent.

(2) An unstable spheroplast-L-form which is able to revert; cell walls are present.

(3) A stable spheroplast-L-form which does not revert, but cell walls are present.

In the present paper we wish to report additional morphological evidence for the the different organization of surface layers in the three types of Proteus L-forms. Electron microscopy of thin sections shows specific stages of deficiency and disorder in cell walls of different L-forms and provides additional support for the L-form classification listed above.

Although thin sections enable the protoplast- and the spheroplast-types of L-forms to be easily distinguished from each other, they are not so useful for localizing the sites of chemical damage within the cell wall which give rise to L-forms. Selective *in situ* identification of individual macromolecular cell-wall components is still not possible in the electron microscope, and it is not known how the different polymer constituents contribute to the image of the thin-sectioned enterobacterial cell wall. Therefore we have studied thin sections of isolated cell walls from normal *Proteus mirabilis* and sections of purified preparations of murein sacculi and lipopolysaccharide. Our findings permit us to draw some conclusions on the location and interaction of wall polymers in normal Proteus cell walls and on the nature and extent of cell wall disorganization in the different Proteus L-forms.

METHODS

The following L-forms and bacterial strains of *Proteus mirabilis* were studied:

(1) The unstable spheroplast-L-form, strain I CA/PEN, was isolated in 1955 by Kandler & Kandler (1956) and re-investigated by Martin (1964). All liquid and solid media in which this organism was cultivated contained Na-benzylpenicillin (Farbwerke Hoechst A. G., Frankfurt/Main-Hoechst) at a concentration of 200 units/ml. Subcultivation of individual spheroplasts of the unstable L-form I CA/PEN on penicillin-

free medium yielded a wide variety of stable L-forms and bacillary organisms (Martin, 1963, 1964). The following descendants of strain 1 CA/PEN were included in this study.

(2) Stable spheroplast-L-form, strains 1 CA/20 and 1 CA/25.

(3) The rod-shaped, non-motile *Proteus mirabilis*, strain 1 CA/5. In addition to this strain normal, rod-shaped *P. mirabilis*, strain VI, was also studied.

(4) Also investigated were the stable protoplast-L-forms, strains LD 52 and LV. These organisms were obtained from Dr U. Taubeneck, Jena. Growth of the protoplast-L-forms required supplementation of the media with 5 % (v/v) defibrinated horse serum.

Culture medium. All organisms used for electron microscopy and for the preparation of cell walls and cell-wall fractions were grown at 35° in aerated liquid cultures with the following composition (g./l.): tryptic casein peptone (E. Merck, A. G., Darmstadt), 15; Lab-lemco beef extract (Oxoid Ltd., London S.E. 1, England), 15; glucose, 2; NaCl, 5; Na_2HPO_4, 4·5; distilled water, 1 l.; pH adjusted to 7·0. The cultures were harvested at the beginning of the stationary phase. The isolation of cell walls and the separation and purification of individual polymer cell-wall components, viz. murein sacculi and lipopolysaccharide, was carried out as described by Martin & Frank (1962) and Martin (1964). An outline of the procedure for the isolation of LPS and murein sacculi is given in Fig. 1.

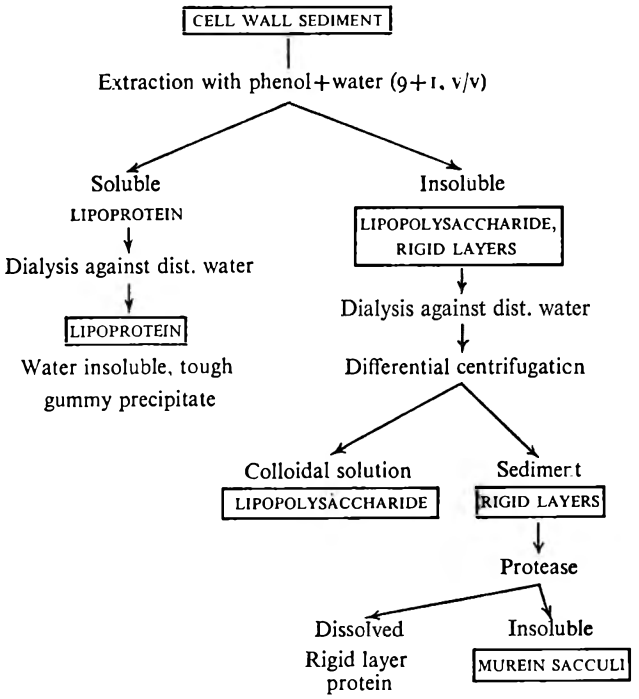

Fig. 1 Fractionation of isolated cell walls of *Proteus mirabilis* into the major macromolecular constituents.

Preparation of specimens. For direct electron microscopy samples were mounted on collodion membranes by the agar filtration technique of Kellenberger (1952) and shadowed with a platinum-iridium alloy. Samples for thin-sectioning were pretreated

in two different ways. Normal bacteria and isolated cell-wall material were fixed with osmium tetroxide (Ryter & Kellenberger, 1958). Potassium bichromate (1 %, w/v) in acetone + water (1/9) was used for the fixation of spheroplasts and protoplasts, since these organisms are easily lysed by osmium tetroxide (Hofschneider, 1960). Bacterial cells were treated in parallel by both methods of fixation in order to make sure that the cell integuments were similarly preserved by the different fixatives. The fixed specimens were embedded in Vestopal W (Ryter & Kellenberger, 1958), sectioned and stained in 0·5 % (w/v) aqueous uranyl acetate (Hofschneider, 1960) and examined in a Siemens Elmiskop I at an original magnification of × 8000.

RESULTS

Phase-contrast microscopy.

This revealed very little difference in gross morphology between the stable protoplast-L-form (Pl. 1, fig. 1), the unstable spheroplast-L-form (Pl. 1, fig. 2) and the stable spheroplast-L-form (Pl. 1, fig. 3). All L-form cultures were assortments of more or less spherical organisms of rather variable size. Reproduction occurred by formation of buds.

Electron microscopy

Thin sections of bacteria and L-forms

Rod-shaped bacteria, strains *Proteus mirabilis* VI and 1 CA/5. A section of the bacterial (rod-shaped) form of *Proteus mirabilis* is shown in Pl. 1, fig. 4, Here, the cell contents are surrounded by two separate and superposed integuments. The inner integument is approximately 80 Å thick and adheres closely to the cytoplasmic cell body. It has the characteristic triple layer structure (electron dense–transparent–dense) of the so-called 'unit membrane' (Robertson, 1959), and thus corresponds to the integument previously described as cytoplasmic membrane in sectioned *Escherichia coli* (Kellenberger & Ryter, 1958). In recent years the term 'unit membrane' has come to imply a standard element of micromorphology and also of physico-chemical cell structure and function (viz. the protein-coated bi-molecular lipid leaflet of the cytoplasmic membrane, harbouring the functions of selective permeability and active transport). In the present paper we prefer the strictly morphological term: Triple Layer integument (TL-integument). This describes a bacterial integument having the appearance but not necessarily the structure and function of 'true' unit membranes, as represented by cytoplasmic membranes and related membraneous organelles. This reservation is particularly pertinent for the description of the outer integument of the entero-bacterial cell which has been assumed to represent the cell wall in studies on thin sectioned *E. coli*. In sections of *P. mirabilis* strain VI, the outer integument was about 100–120 Å thick but otherwise resembled the inner one; it was also a TL-integument, although in some places a third electron-dense layer seemed to be present, bringing the total to five layers: dense–transparent–dense–transparent–dense. Recently, several authors have published micrographs of *E. coli* sections in which three electron-dense layers can be clearly distinguished in the outer integument (Frank & Dekegel, 1965; Murray, Steed & Elson, 1965; de Petris, 1965; Weibull, 1965). In some cases these structures resemble the familiar picture of two merging unit membranes (Robertson, 1959). Frank & Dekegel have re-investigated sections of our Proteus strain 1 CA/5 and VI and have found five-layered outer integuments in some cases (Frank & Dekegel,

1967). However, this feature was not consistently observed. Triple-layered outer integuments were found in other cells of the same preparation.

Stable protoplast-L-form, strains LD 52 and L VI. In sections of strains LD 52 and L VI only one integument was observed, a TL-integument located immediately outside the cytoplasm (Pl. 1, fig. 5). The identity of this TL-integument with the cytoplasmic membrane is suggested by its sensitivity to mechanical and osmotic stress and the action of detergent. By osmotic shock of L-form organisms in distilled water separate integuments were obtained as empty 'ghosts'. Metal-shadowed preparations of these structures disintegrated rapidly into smaller fragments or coalesced into larger aggregates under the electron beam. In dilute aqueous solutions of sodium dodecylsulphate (0·05 %, w/v) the ghosts were instantly dissolved without leaving any particulate residue visible in the electron microscope (Martin, unpublished observations).

Stable spheroplast-L-form, strains ICA/20 (Pl. 1, fig. 6) and ICA/25. These organisms were found to have two separate, superposed integuments of virtually identical appearance. Both were TL-integuments of very similar thickness: approximately 80–100 Å. The inner TL-integument was located in the same position as the presumed cytoplasmic membrane of the protoplast-L-form; the outer TL-integument was in a corresponding position to the cell wall of normal *Proteus mirabilis* cells.

Unstable spheroplast-L-form, strain ICA/PEN, grown in the presence of penicillin (Pl. 2, fig. 7*a*). An outer and an inner integument were observed. Only the inner one was a perfect TL-integument, resembling the 'cytoplasmic membrane' of the other L-forms and the bacterial form. The outer integument (cell wall) showed signs of extensive disorganization; only short, intermittent stretches of TL-integument were preserved, connected by stretches of diffusely contoured single-layer integument. Other cell-wall material was seen in the process of detachment from the outer surface of the cell wall. Massive dissociation of small, globular particles from the outside of the same L-form spheroplasts had been found previously in metal-shadowed preparations (Martin, 1964) and is shown again in Pl. 2, fig. 7*b*. In the thin-sectioned L-form preparation (Pl. 2, fig. 7*a*) one can see that the surface particles are hollow spherules bordered by single TL-integuments.

Thin sections of isolated cell walls and cell-wall polymers of rod-shaped Proteus bacteria

Cell walls. Thin sections of isolated and thoroughly washed cell walls of *Proteus mirabilis*, strain ICA/5 (Pl. 2, fig. 8), revealed structures resembling well preserved TL-integuments, although the distinct frayed appearance of the two electron-dense layers is atypical. Otherwise, this observation supports the previous conclusions about sections of whole bacteria and spheroplast-L-forms. It should be explained, however, why a sectioned cell wall displays an ultrastructure which is thought to be characteristic for cytoplasmic membranes and related membraneous organelles. One possible explanation, contamination of the isolated cell walls with cytoplasmic membrane matter, seems to be excluded. Cell wall isolation involved vigorous and prolonged shaking of bacteria with glass beads in aqueous suspension containing 0·8 % (w/v) sodium dodecylsulphate. As cytoplasmic membranes of the stable protoplast-L-form are dissolved instantly by much smaller detergent concentrations, it is unlikely that cytoplasmic membrane residues adhering to the cell wall could have survived the combined mechanical treatment and detergent extraction.

Cell-wall polymers. Not all of the major polymer components of the enterobacterial cell wall could be isolated in a purified state which is amenable to the thin-sectioning technique. The lipoprotein fraction was obtained as a tough, rubber-like substance, unfit for processing on the ultramicrotome. Useful thin-sections could be prepared from isolated lipopolysaccharide and murein sacculi.

Lipopolysaccharide. In metal-shadowed specimens of Proteus LPS (Pl. 2, fig. 9) we saw typical vermiform particles with a tendency to merge into larger platelets, quite in analogy to previously described lipopolysaccharides of a Salmonella species (Schramm, Westphal & Lüderitz, 1952) and *Escherichia coli* (Weidel *et al.* 1960). In thin-sections the LPS-particles were revealed as hollow bags or tubes which were again bordered by TL-integuments (Pl. 3, fig. 10). The spacing and dimensions within the LPS-triple layer were very similar to those found in sections of whole cell walls, with which sections of larger LPS-aggregates might therefore be confused.

Murein sacculi. Isolated murein sacculi of *Proteus mirabilis*, strain 1 CA/5 have the well-known appearance of the 'bag-shaped macromolecules' which determine the form of the bacterial cell (Pl. 3, fig. 11). In sacculus sections (Pl. 3, fig. 12) the profile of the murein appears as a single electron-dense line with an approximate thickness of 20 to 25 Å. The lower value probably applies to those areas where the murein sheet has been cut most nearly at a right angle.

Sections of isolated cell walls after removal of murein with lysozyme. Since it has been shown that sectioned murein and LPS have typical morphological features with definite dimensions, one would now like to know whether or not electron microscopy can tell us something about the position of these substances, relative to each other, within the cell wall. Thus, for instance, it should be possible to localize the site of the murein sacculus by comparing thin-sections of normal isolated cell walls with those of cell walls from which murein has been specifically removed by the action of lysozyme. Isolated cell walls of *Proteus mirabilis*, strain 1 CA/5 (50 mg dry weight) were incubated for 16 hr at 37° in 20 ml. 0·1 M-tris buffer, pH 7·0, containing 300 μg. crystalline egg-white lysozyme (Worthington Chem. Co., Freehold, N.J., U.S.A.). Soluble murein fragments were removed from the digested cell walls by four consecutive washings with tris buffer and distilled water and sedimentation of the residual cell walls in the centrifuge at 20,000 g. In thin sections of lysozyme-treated cell walls (Pl. 3, fig. 13) the original cell shape was fairly well preserved. The walls also retained their organization as TL-integuments with virtually the same dimensions as in untreated cell walls. No disappearance of an electron-dense 20 Å layer from the TL-integument could be observed. On the other hand, the enzymic breakdown of the murein sacculus had a very marked effect on the mechanical stability of the cell wall and its sensitivity to the action of detergents. Now, shaking with glass beads for a few seconds caused complete fragmentation, and incubation in a solution of 0·4 % (w/v) sodium dodecylsulphate induced disaggregation of the cell walls into small particles.

DISCUSSION

Electron microscopy of thin-sectioned Proteus L-forms leaves no doubt that this group of 'cell wall-defective bacteria' (Davis, 1966) contains organisms of the spheroplast-type as well as forms which are virtually indistinguishable from bacterial protoplasts. Concerning the protoplast nature of the stable protoplast L-form our observa-

tions agree well with the previous reports of Weibull (1965) and Tulasne, Minck & Kirn (1962). In Proteus the ability to form a cell wall must be closely linked with the ability to construct a murein sacculus. It has been observed earlier that the stable protoplast L-form strains LVI (Martin, 1964) and LD52 (Weibull, 1965) produce LPS. Moreover, it has been shown that LVI produces functional bacteriophage receptor substances and excretes them into the culture medium (Zickler, 1967), although the L-form-protoplasts themselves are phage-resistant. It must be concluded, that although plastic wall polymers such as LPS and probably also lipoprotein are synthesized by the protoplast L-form, the protoplasts are not able to retain these substances on their surfaces and integrate them into a coherent integument in the absence of murein. In the stable and unstable spheroplast L-forms our observations on the presence of cell walls in thin sections agree well with the previous reports on the occurrence of murein and other typical wall materials in these organisms (Kandler *et al.* 1958; Martin, 1964). But the decreased thickness of the L-form walls and the visible escape of wall material from the spheroplast surface of the unstable L-form indicate that here the plastic wall polymers cannot be as tightly bound to their murein base as in the normal bacterial form. We know from previous studies that the LPS content of cell walls decreases from 42 % in normal *P. mirabilis*, strain VI, to 24 % in the stable spheroplast-L-form, strain 1 CA/25, and 11 % in the unstable spheroplast-L-form, strain 1 CA/PEN (Martin, 1963). The loose adhesion of the plastic wall polymers may be largely due to the defective architecture of the murein sacculi in spheroplast-L-forms (Martin, 1964, 1967). However, another explanation is also possible. It has been shown that the component of the enterobacterial cell wall which determines shape does not exist in the form of a simple murein sacculus, but rather as a so-called 'rigid layer', a covalently interlinked complex of a murein sacculus and protein. In 'rigid layers' of normal, rod-shaped *Escherichia coli* and *Proteus mirabilis* the protein is attached to the surface of the sacculi as a multitude of tuft-like appendages, giving the appearance, under the electron microscope, of a regular, granular surface layer (Weidel *et al.* 1960; Martin, 1964). Recent observations by Fishman & Weinbaum (1967) on negatively stained *E. coli* walls suggest that rigid-layer protein is even more regularly attached to the murein sacculus than has so far been suspected. In contrast, the rigid layer equivalents of the L-form cell walls lack this regular arrangement of the protein component. In the heavily damaged structures, which in the unstable spheroplast L-form correspond to the rigid layer, the protein content is also greatly reduced (Martin, 1964).

One might assume that rigid layer protein normally serves to attach lipoprotein and lipopolysaccharide to the cell wall. In its disorganized state in the L-form cell wall it may no longer be able to fulfil this function. A similar interpretation might also apply to the recently reported spontaneous dissociation of LPS from the cell walls of a lysine-requiring *Escherichia coli* mutant (Knox, Vesk & Work, 1966). In summary, we should like to propose a schematic view of the profile of a normal enterobacterial cell wall (Fig. 2), and its state in the unstable spheroplast L-form of *Proteus mirabilis* (Fig. 3). These models also take into account the previous studies on the composition of the cell wall of *E. coli* B (Weidel, Koch & Lohss, 1954; Weidel, Koch & Bobosch, 1954; Weidel & Kellenberger, 1955; Weidel, 1958; Weidel *et al.* 1960). This work has shown that lipoprotein and lipopolysaccharide are deposited on the outer surface of the 'rigid layer', making the murein sacculus within the intact cell-wall complex inaccessible to the attack of lytic enzymes from without. Both lipoprotein and LPS display their

functions as antigens and bacteriophage receptors on the cell surface. However, quantitative phage receptor tests have shown that a large part of potential receptor sites in the lipopolysaccharide (for phages T3, T4, and T7) are masked, probably by partially superposed lipoprotein. Plastic wall polymers have been extracted from the cell wall of *E. coli* B in the form of minute globules displaying specific receptor function for bacteriophage T5. In these isolated receptor particles LPS has been found to form the core and lipoprotein the superposed surface coating. More recently, T5-receptor particles have been studied as thin sections and have been shown to be hollow

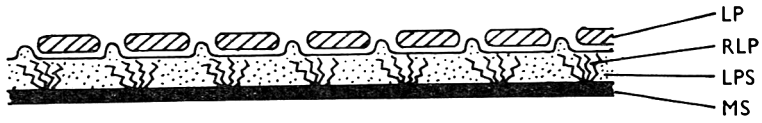

Fig. 2. Schematic view of cross-sectioned cell wall of normal rod-shaped *Proteus mirabilis*. LP = lipoprotein; LPS = lipopolysaccharide; MS = murein sacculus; RLP = rigid layer protein. Explanation in text.

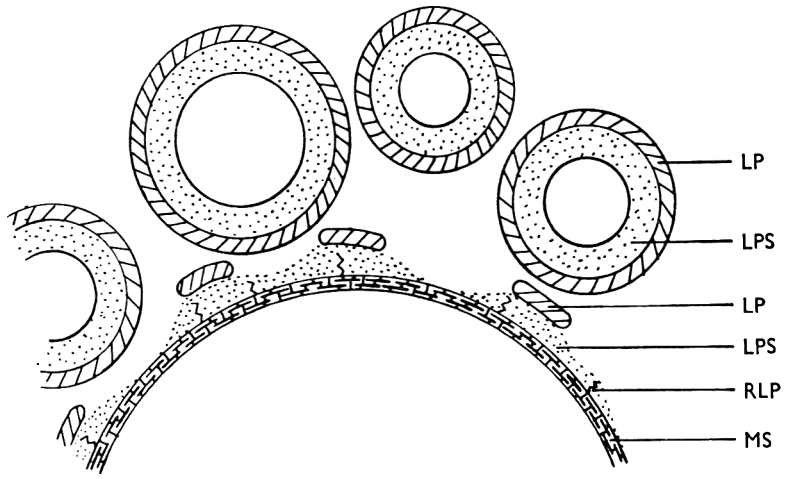

Fig. 3. Cross-section of the damaged cell wall of unstable L-form spheroplast, schematic view Abbreviations as in Fig. 2. Reduced stability of the murein sacculus and extensive loss of rigid layer protein allow the plastic wall material LP and LPS to leave the cell wall in the form of hollow spherical particles.

bags surrounded by TL-integument (Frank & Dekegel, 1965, 1967). The sections of T5-receptor closely resemble those of the spherules which are liberated spontaneously from the spheroplast surface of the unstable Proteus spheroplast-L-form. The TL-integuments of both of these surface particles are indistinguishable from the TL-integument of isolated LPS from *E. coli* (Frank & Dekegel, 1965, 1967) and *P. mirabilis*. Our studies and those of Frank & Dekegel (1965, 1967) have shown clearly that two of the major constituents of enterobacterial cell walls, LPS and murein, have well-defined morphological properties when sectioned and viewed as isolated preparations. However, these observations are still of limited value for establishing the sites and space requirements of LPS and murein in the intact cell wall. When sections of intact isolated cell walls and LPS are compared, it is obvious that much, if not most, of

the cell wall image is contributed by the LPS alone. Some, but certainly not all, of the lipoprotein may have been dissolved during the isolation of cell by the mechanical treatment and detergent extraction. Even so, one cannot localize the residual lipoprotein and the rigid-layer in the TL-integument of the cell-wall section. Therefore it seems necessary to conclude that it is not possible with the techniques now available to identify the lipoprotein and the rigid layer within sectioned cell walls as structures distinct from the LPS. Other questions must also be left unanswered. Thus, the basis for the TL-integument formation by the macromolecular LPS is far from clear. The structural principle could hardly be the same as in the formation of the bi-molecular leaflet from low-molecular weight lipid molecules in cytoplasmic membranes. Cell walls of enteric bacteria (just as those of Gram-positive bacteria) have been found to act as molecular sieves (Lieve, 1965*a*, *b*; Polsinelli, Ciferri, Cassani & Albertini, 1964), but they are not known to have properties of selective permeability similar to those of cytoplasmic membranes. On the contrary, in thin sections of plasmolysed *E. coli* as in plant cells, it can be seen that the cell content within the cytoplasmic membrane decreases in size and recedes from the cell wall which retains its normal size and shape (Cota-Robles, 1963). In the studies of Frank & Dekegel (1965, 1967) and also in our work the thickness of sectioned murein sacculi in *E. coli* and in *P. mirabilis* has been determined at 20–25 Å. If these values should prove to be correct they would lend support to the recently proposed assumption that murein sacculi of enterobacterial cell wall are monolayers of murein (Weidel & Pelzer, 1964).

We are greatly indebted to Professors A. Butenandt and O. Kandler for their generous support and continued interest in this work. The capable and diligent assistance of Miss Anneliese Preuss in the electron microscopical work is gladly acknowledged. Our study was supported by grants of the Deutsche Forschungsgemeinschaft to H. H. Martin and P. H. Hofschneider.

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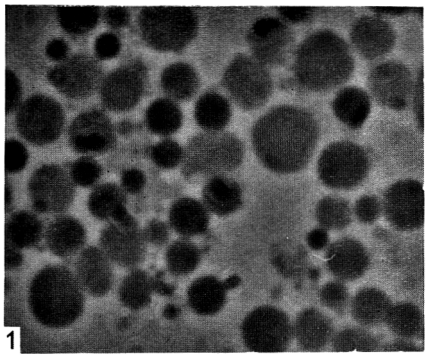
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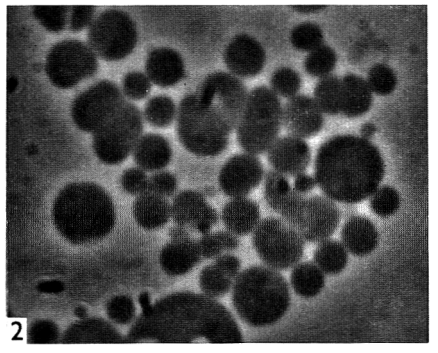
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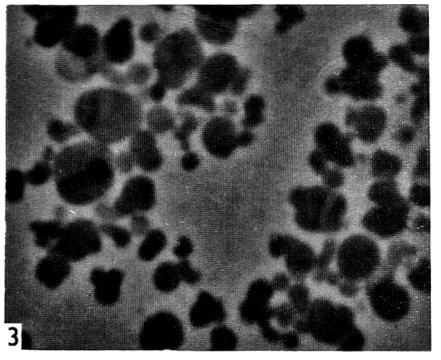
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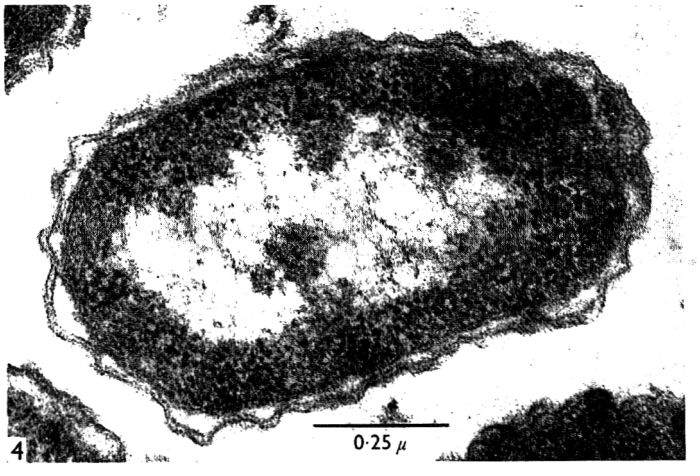
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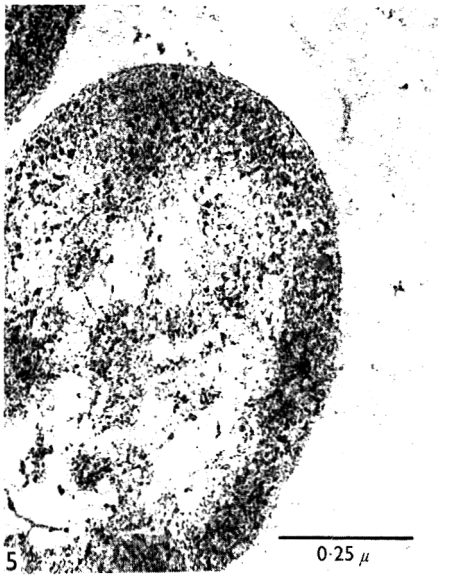
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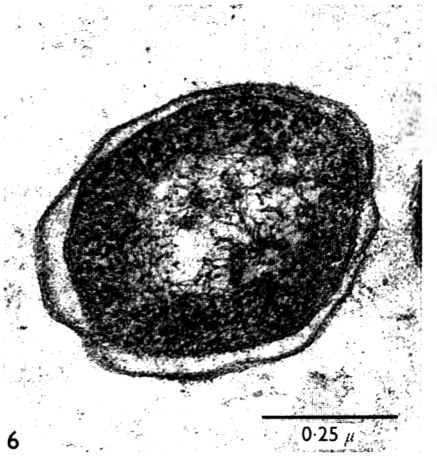
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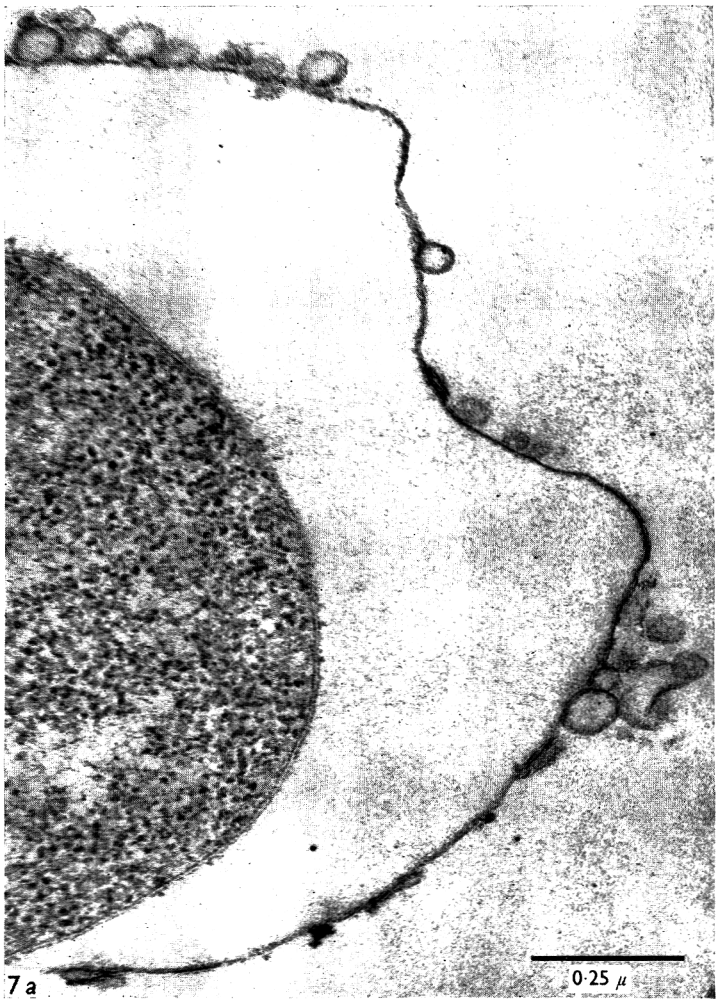

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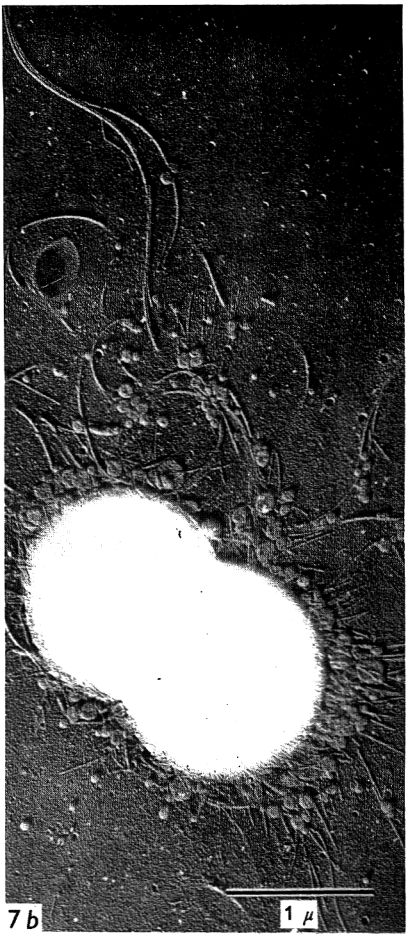

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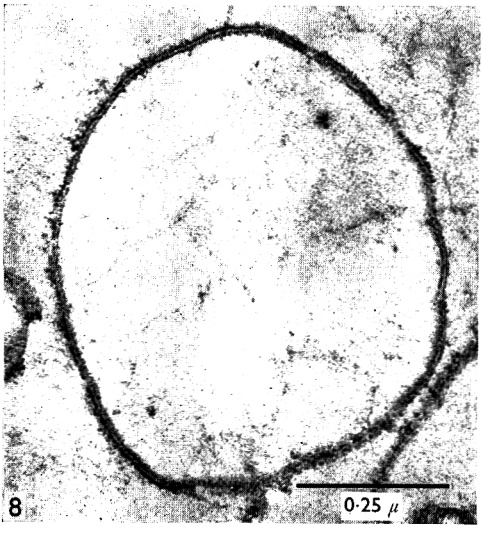

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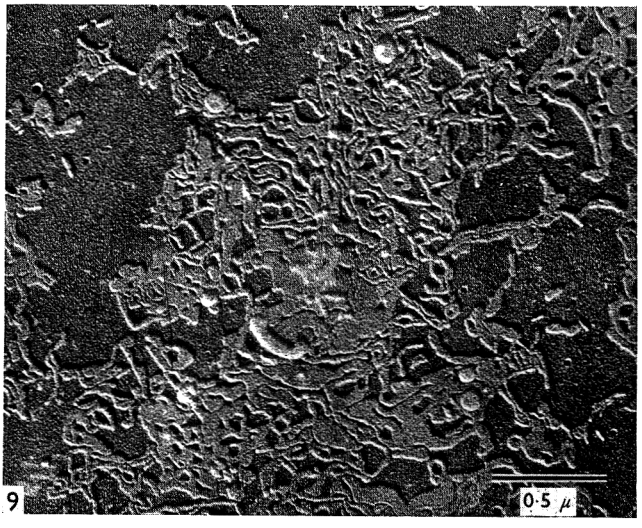

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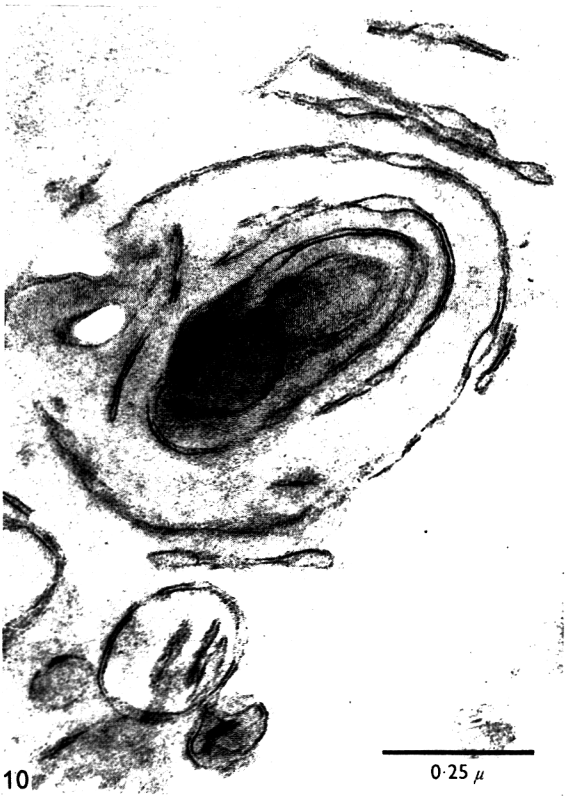

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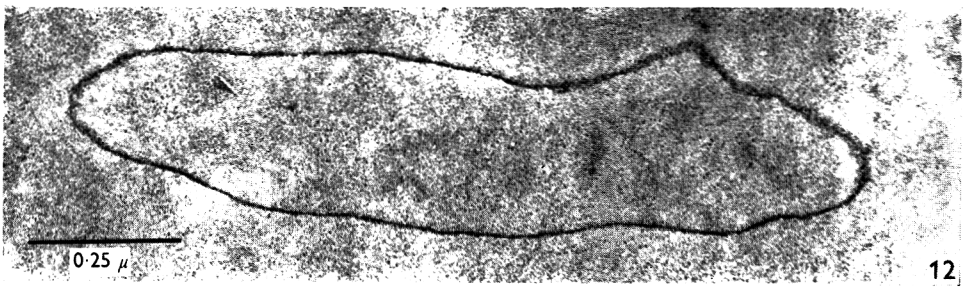

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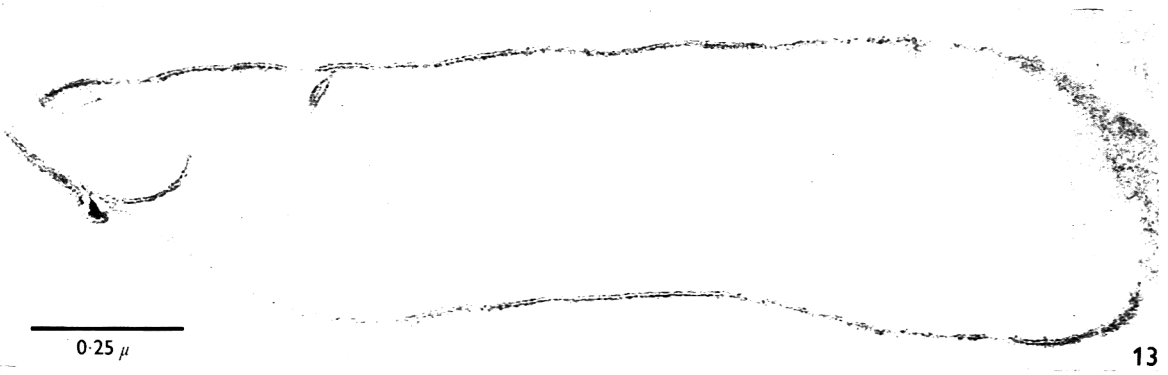

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Stable protoplast L-form of *Proteus mirabilis*, strain LD 52; phase-contrast. × 3600.

Fig. 2. Unstable spheroplast L-form of *Proteus mirabilis*, strain 1 CA/PEN; phase-contrast. × 3600.

Fig. 3. Stable spheroplast L-form of *P. mirabilis*, strain 1 CA/20; phase-contrast. × 3600.

Fig. 4. Section of rod-shaped *P. mirabilis*, strain VI. × 80,000.

Fig. 5. Section of stable protoplast L-form, strain LD 52. × 80,000.

Fig. 6. Section of stable spheroplast L-form, strain 1 CA/20. × 80,000.

PLATE 2

Fig. 7. Unstable spheroplast L-form, strain 1 CA/PEN: (*a*) section of L-form spheroplast. × 80,000 (*b*) Pt-Ir-shadowed L-form spheroplast in the process of division. × 19,300.

Fig. 8. Section of isolated cell wall of rod-shaped *P. mirabilis*, strain 1 CA/5. × 80,000.

Fig. 9. Lipopolysaccharide, isolated from cell walls of *P. mirabilis*, strain 1 CA/5. Pt-Ir-shadowed. × 30,000.

PLATE 3

Fig. 10. Section of isolated lipopolysaccharide. × 80,000.

Fig. 11. Murein sacculus, isolated from cell wall of *P. mirabilis*, strain 1 CA/5. Pt-Ir-shadowed. × 18,000.

Fig. 12. Section of isolated murein sacculi of *P. mirabilis*, strain 1 CA/5. × 80,000.

Fig. 13. Section of isolated cell wall of rod-shaped *P. mirabilis*, *strain* 1 CA/5, after removal of murein by lysozyme treatment. × 80,000.

The bars on Pl. 1, figs. 4–6, Pl. 2, figs. 7*a* and 8 and Pl. 3, figs. 10, 12 and 13 represent 0·25 μ. The bar on Pl. 2, fig. 9 represents 0·5 μ. The bars on Pl. 2, fig. 7*b* and Pl. 3, fig. 11 represent 1 μ.

J. gen. Microbiol. (1968), **51**, 35 42
With 3 plates
Printed in Great Britain

Distinction between Bacterial and Algal Utilization of Soluble Substances in the Sea

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(*Accepted for publication* 1 *September* 1967)

SUMMARY

The presence of numerous bacteria and diatoms attached to the sand grains of a littoral beach have been shown by fluorescence microscopy. Bacteria and diatoms were found in a viable condition to depths exceeding 10 cm. The rate of uptake of [^{14}C]-acetate was measured over the range 10–5000 μg./l. and the results analysed by Michaelis–Menten kinetics. By the use of autoradiography it was shown that the bacteria alone were responsible for the uptake of [^{3}H]-acetate. It is concluded that algal heterotrophy is negligible in sea waters.

INTRODUCTION

The available substrate for heterotrophy in the sea comprise both particulate and soluble organic material, the latter predominating by a factor of ten or more (Parsons & Strickland, 1962). The soluble material is made up of heterogeneous compounds of which only a fraction is likely to contain compounds of low molecular weight. Reliable reports of the concentration of individual compounds are few; however, Siegel & Degens (1966) have reported a mixture of 17 free amino acids constituting 66 μg./l. in Buzzards Bay, Cape Cod, and Degens, Reuter & Shaw (1964) reported glucose, galactose and mannose concentrations of 3–18 μg./l. (10^{-8} to 10^{-7}M) in offshore Californian seawaters. Wright & Hobbie (1965, 1966) and Vaccaro & Jannasch (1966) described the uptake of single substrates by pure cultures of bacteria at concentrations in excess of 10^{-8}M. In a similar survey of four pelagic species of algae, including one known heterotroph, Sloan & Stickland (1966) concluded that uptake at concentrations of 0·25 mg. C/l. (5×10^{-6}M) was insufficient to balance even respiratory requirements.

The low concentration of suitable substrates dictates that measurement of heterotrophy within a natural population requires the development of special techniques. In measuring the 'relative heterotrophic potential' of sea waters Parsons & Strickland (1962) were the first to use a [^{14}C]-labelled organic substrate in a manner analogous to the [^{14}C]-carbonate technique developed by Steeman Nielsen (1952) for marine photosynthesis. Both these authors and Wright & Hobbie (1965, 1966) showed that the rate of uptake of substrate by heterotrophic organisms within an aquatic population followed a typical saturation curve which could be analysed by the Michaelis–Menten equation at low concentrations ($> 10^{-6}$M). At substrate concentrations $< 10^{-6}$M,

much higher than exist in natural waters Wright & Hobbie (1965, 1966) found that both fresh water lake populations and pure cultures of algae isolated from these lakes showed a second uptake mechanism based on diffusion kinetics and concluded that the algae present in the natural population were responsible for most of the uptake. Other authors (Rodhe, 1955; Wood, 1956) have concluded from direct observations that algal cells in a natural community deprived of light do resort to heterotrophy.

The present paper sets out to measure the rate of uptake of a single organic substrate by a natural population and to determine if the algae present contributed to such uptake.

METHODS

The experiments outlined below were made with naturally occurring microbial populations attached to sand grains. Sand samples were taken from the littoral zone of an exposed sandy bay in a sea loch, Loch Ewe, on the west of Scotland.

Uptake experiments. The methods used were essentially those described by Wright & Hobbie (1965), adapted to suit sand populations. Experiments were made by incubating duplicate samples of 10 g. sand + 25 ml. filtered (Whatman GF/C grade) seawater in 60 ml. Pyrex glass-stoppered bottles. Incubations were carried out at the prevailing sea-water temperature (10–14°) in dark incubators. High specific activity uniformly labelled [^{14}C]-acetate (Radiochemical Centre, Amersham, Buckinghamshire) was prepared in sterile ampoules and added in μl. amounts at the start of incubation. Zero time controls were prepared by adding neutralized formalin immediately after addition of the isotope. Samples were shaken twice during the course of 1-hr incubations. Metabolism was stopped by adding formalin, the sand filtered off and washed with filtered sea-water. The sand samples were stored at $-15°$ and dried before counting on aluminium planchettes in an end-window counter of known efficiency. Self absorption of radiation due to the sand was allowed for by multiplying counts/min. by a factor of 21·5 (Baird & Wetzel, 1967).

Material for autoradiography. Sand samples were prepared by incubating 2 g. sand + 10 ml. filtered seawater in light or dark incubators with [^{14}C]-carbonate or uniformly labelled [^{3}H]-acetate, respectively. Other conditions were as previously described except that light incubations were for 5 hr at 1000 ft.c. and at the end of incubation all sand samples were washed with distilled water. Washed sand samples were treated for 20–30 sec. at 20 kcyc./sec. in an ultrasonic disintegrator, then two drops of supernatant fluid were spread immediately over the surface of a slide treated with Ullrich's fixative, dried and washed.

Slides were dipped in Kodak NTB–2 liquid emulsion (diluted 1/2) held at 45°, and exposed for 3–7 days. Detailed methodology for the use of autoradiography with micro-organisms in aquatic habitats will appear elsewhere (Brock & Brock, 1967). The photographs of autoradiograms were taken on Polaroid Type 42 film with a Polaroid MP-3 camera.

RESULTS

The microbial population. The microbial population attached to the sand grains in question was found by fluorescent microscopy to be principally composed of diatoms (Bacillariophyta; Pl. 1, fig. 1–3) and bacteria (Pl. 2, fig. 4–6). Samples were taken at low tide where both populations extend to a depth of more than 15 cm. Additional evidence of diatoms at these depths was shown by chlorophyll and chlorophyll

phaeopigment analyses carried out on vertical profiles of the beach by Steel & Baird (1967). The high ratios of chlorophyll to phaeopigment found by these authors as well as uniform uptake of $^{14}CO_2$ throughout the profiles suggest algal cells in good physiological condition. However, light penetration is only 1% at 3 mm. depth in quartz sand and studies of sand mixing indicate that only the top 5 cm. regularly moves under wave action. Therefore much of the algal population may be in total darkness for periods up to many months. The situation of these algae would seem analogous to that described by other authors (Rodhe, 1955; Wood, 1956) who found algal cells apparently in good physiological condition yet living in total darkness under thick ice or at considerable depths in the oceans.

The bacterial population was readily detectable to a depth of 15 cm. as was shown by viable counts of $0{\cdot}5\text{–}3 \times 10^5$/g. sand and by staining the sand with acridine orange (Pl. 2, fig. 4–6,). If the number of objects attached to the sand grains which fluoresce green are truly all viable bacteria then the viable counts represent 0·1% or less of the total population.

Kinetic analyses. An equation for uptake of a [^{14}C]-substrate by an active population was first proposed by Steeman Nielsen (1952) and further modified by Parsons & Strickland (1962)

$$v = \frac{cf(Sn+A)}{C\,\mu t,} \tag{1}$$

where v is the velocity of substrate uptake (mg. l.$^{-1}$ hr^{-1}), c is the radioactivity contained in the population, Sn is the concentration (mg. l.$^{-1}$) of a given substrate present in the natural sample and is assumed to be of negligible proportions compared to the added substrate, A the concentration (mg. l. $^{-1}$) of added substrate, C the counts min.$^{-1}$ from 1 μc [^{14}C] in the counting assembly used, μ the number of microcuries added to the sample bottle, t the incubation time (hr), and f is a factor to compensate for any discrimination between [^{14}C] and [^{12}C] atoms, which in the present experiments is neglected. In the present experiments c is the radioactivity associated with the sand grains and is multiplied by a factor of 21·5 (Baird & Wetzel, 1967) to allow for self-absorption of radiation by the sand grains and v, the velocity, is expressed as mg. g.$^{-1}$ sand hr^{-1}.

The results of measuring the velocity of [^{14}C] uptake from uniformly labelled acetate are shown from four different months in Fig. 1. It can be seen that all the curves resemble each other in sharply increasing velocities at low concentrations and thereafter by a decrease in rate. As all metabolism was terminated by addition of formalin it is most likely that all the [^{14}C] measured as uptake was no longer acetate but part of the particulate fraction of the cells in question.

The rate of uptake (v) has been shown by Parsons & Strickland (1962) to follow a saturation curve or Langmuir isotherm. If the uptake rate is proportional to time as well it is possible to apply the Michaelis–Menten equation to elucidate some of the properties of the natural population. Figure 2 shows that uptake at two substrate concentrations was proportional to time over 1 to 1·5 hr periods.

Conversion of the velocity curve into a linear form using a modified Lineweaver–Burke equation gives

$$\frac{Sn+A}{v} = \frac{K_t+Sn}{V} + \frac{A}{V}, \tag{2}$$

where v is the velocity of uptake at each concentration of substrate tested, V the maximum rate attainable and K_t is a constant. Substituting from (1) gives

$$\frac{C\mu t}{c} = \frac{K_t + Sn}{V} + \frac{A}{V}. \tag{3}$$

Using equation (3) a plot of $C\mu t/c$ versus A can be used to solve for $K_t + Sn$, the intercept of the straight line on the x-axis and V, the inverse of the slope of the straight line. The intercept on the ordinate, T_t (hr), gives a measure of the turnover time of the natural substrate (Wright & Hobbie, 1966).

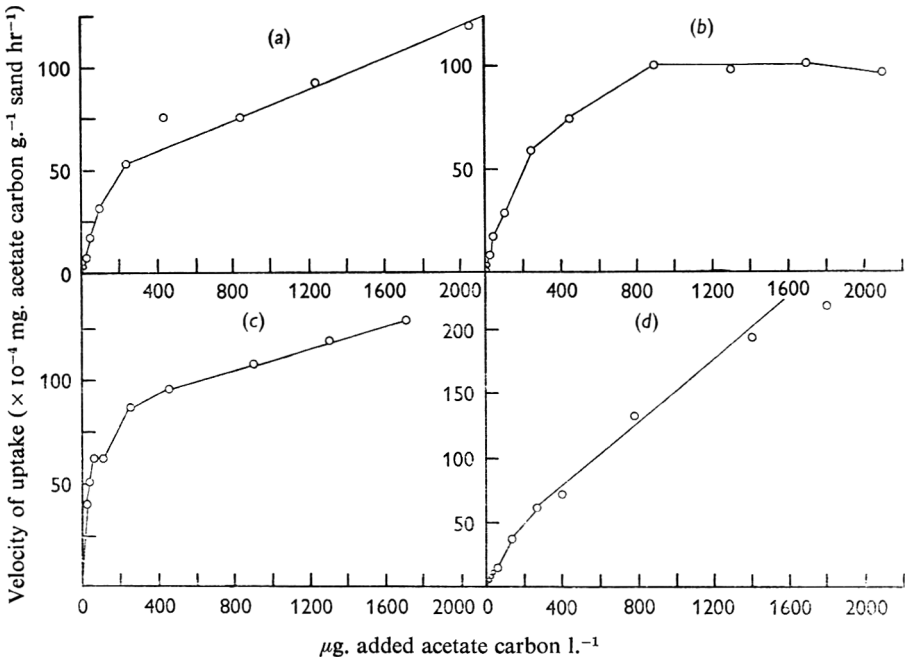

Fig. 1. Uptake velocity versus substrate concentration from four different months 1966. Sand from 10 cm. depth, from a low-water station. (*a*) 23 March at 10°, (*b*) 18 May at 11°, (*c*) 27 July at 12·5°, (*d*) 10 September at 14°.

The result of plotting $C\mu t/c$ against a limited range of concentrations of A is shown for the 4 months in Fig. 3. Only on two occasions, 18 May and 27 July, could all the constants be satisfactorily solved. For 23 March and 10 September the straight line is nearly parallel to the x-axis, implying that rate was proprotional to concentration. The collected results for the parameters evaluated are shown in Table 1. The values of V were much greater than any of the reported results for water, a finding which can be attributed to the much larger populations in a g. of sand than in 1 l. of sea water.

The second uptake mechanism described by Wright & Hobbie (1965, 1966) in planktonic populations showed a linear increase in uptake velocity at increasing substrate concentrations. The slope of this line, kd (hr^{-1}), was found to be identical to a similar constant derived from the kinetics of simple diffusion. On three occasions, 23 March, 27 July and 10 September, our results allowed the calculation of such constants, which are recorded in Table 1.

Wright & Hobbie (1966) attempted to distinguish kinetically the radioactive component taken up by algae as compared to bacteria. Autoradiography (Brock & Brock, 1966) provides a direct means of distinguishing bacterial from algal uptake. We have

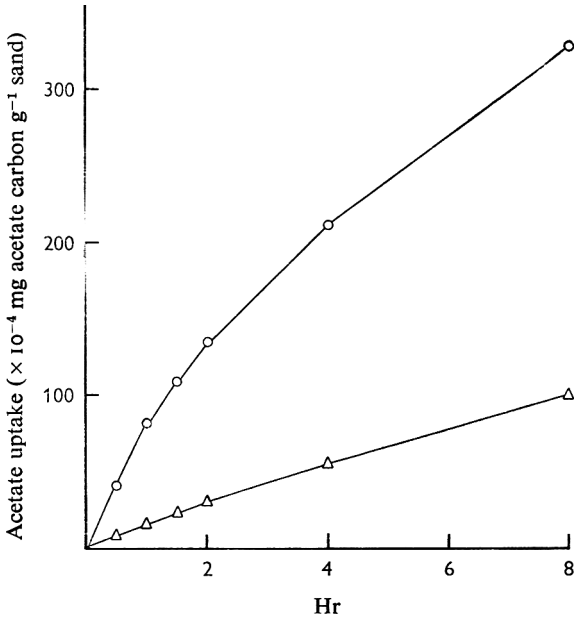

Fig. 2. Uptake at △ = 48 μg. and ○ = 848 μg. acetate-C over an 8 hr period on 22 March 1966. Temperature of incubation 10°. Sand from 10 cm. depth from a low water station.

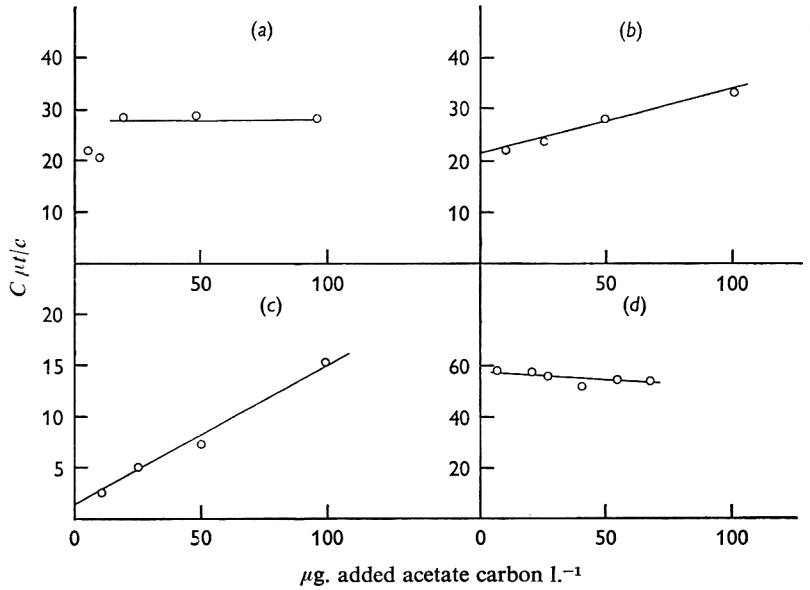

Fig. 3. Data from Fig. 1. plotted by modified Lineweaver–Burke equation showing relationships between $C\,\mu t/c$ and added acetate.

prepared autoradiograms of material incubated in various concentrations of [^{3}H]-acetate and [^{3}H]-glucose. We have also prepared autoradiograms of material incubated with $^{14}CO_2$ in the light.

Table 1. *Collected kinetic data using naturally occurring microbial populations attached to sand grains*

Dates	23. iii. 1966	18. v. 1966	27. vii. 1966	10. ix. 1966
V (mg. acetate C $\times 10^{-4}$):	—	80	71·5	—
$Kt+S$ (μg. acetate C):	—	170	10	—
T_t (hr)	—	21·5	1·5	—
kd (hr $\times 10^{-4}$):	36·5	—	26	122

At all concentrations of acetate or glucose, the diatoms were unlabelled, whereas many labelled bacteria and bacterial microcolonies were seen. Plate 3, fig. 7 and 8 show two labelled bacterial clusters adjacent to unlabelled diatoms when a concentration of 330 μg. acetate/l. was used, while Pl. 3, fig. 9 and 10 show similar situations when 990 and 330 μg.acetate/l. were used. The diatoms in the original preparations were viable, as shown by the fact that they became labelled when incubated in the light with $^{14}CO_2$. Two labelled diatoms are seen in Pl. 3, fig. 11 and 12.

The results illustrated in the photographs are representative of those seen in a large number of microscope fields. In no case did any diatoms beome labelled when organic substrates were used.

DISCUSSION

From these results we can conclude that the assimilation of organic materials was by the bacteria associated with the sand, even when high concentrations were used. We assume that the bacteria stripped from the sand grains by brief ultrasonic treatment, and hence detected autoradiographically, were typical of those seen by fluorescence microscopy attached to the sand. The large numbers of bacteria seen on the sand particles should be emphasized. Only by reflected fluorescence microscopy can these organisms be readily seen, and virtually every sand particle observed was well colonized. Diatoms were also present on most sand particles, but seemed to be present in lower numbers than bacteria. However, Steel & Baird (1967) found a high correlation between the chlorophyll and carbon content of this sand, suggesting that the algal biomass constituted most of the organic material present.

By use of the Wright & Hobbie technique it was possible to calculate diffusion constants. From this and the evidence of a large algal biomass it might have been concluded that uptake of organic substances at higher concentrations was algal. Autoradiography shows that this conclusion is not justified, and emphasizes the danger of attempting to determine the presence of two components of a process by kinetic data alone. The reason for altered kinetics at higher substrate concentrations is obscure, but autoradiography shows that bacteria were responsible for all the uptake measured and hence the shape of the velocity versus concentration curves. On two of the four occasions it was possible to evaluate V and K_t+Sn suggesting kinetics compatible with the Michaelis–Menten equation. On the other two occasions Fig. 3*a*, *d* show a zero-order reaction, a result also found by Wright & Hobbie (1965) for some natural populations. The reasons for these findings remain obscure.

In aquatic environments organic concentrations are generally low, although in the

present case absorption phenomena may have led to concentrations on the sand higher than those in the free water. Bacteria are clearly able to compete successfully for the limited organic matter available, and it seems reasonable to generalize this conclusion to other aquatic situations. Aquatic bacteria have undoubtedly evolved for growth on low organic concentrations, and it seems likely that any small molecular weight organic substances which become available in the environment will be preferentially assimilated by them.

The diatoms living below the photic zone are viable as shown by autoradiography of cells exposed to [^{14}C]-carbonate in the light and also by the results of Steel & Baird (1967) on [^{14}C] measurements on vertical profiles. The viability of these diatoms cannot be explained by heterotrophy. A parallel between this environment and finding algae in deep oceanic waters suggests that a low level of endogenous metabolism may play an important part in the survival of algal cells which had otherwise been assumed to resort to heterotrophy. In closing, we might point out that autoradiography is a powerful tool in the study of energy and trophic relations in microbial ecosystems.

We wish to acknowledge the indispensable assistance of M. Louise Brock in the autoradiography experiments. T. D. Brock is a Research Career Development Awardee of the U.S. Public Health Service (AI-K3-18, 403), and his research is supported in part by a grant from the U.S. National Science Foundation (GB-5258).

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–3. Transmitted ultraviolet illumination showing fluorescing chloroplasts within diatoms.

Fig. 1. A sand grain from 8 to 10 cm depth. 2 May 1966. Sample taken at a low-water station.

Fig. 2. Sand grains from 0 to 1 cm depth. 2 May 1966. Sample from sublittoral beach.

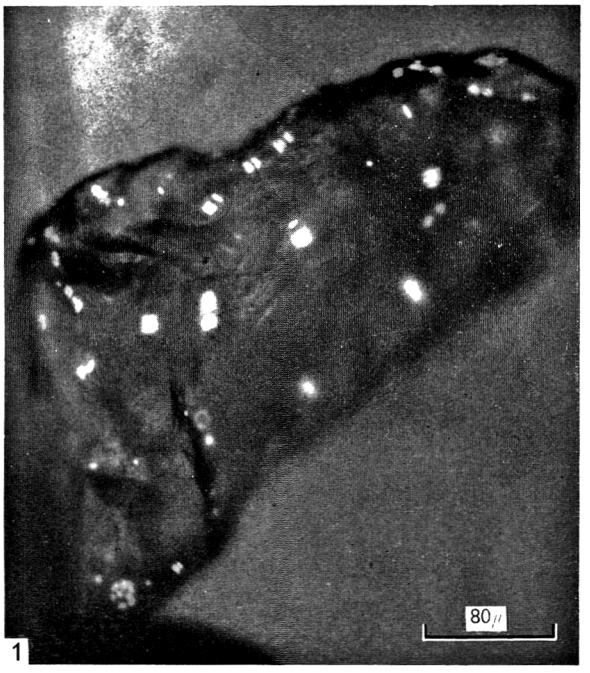
Fig. 3. Sand grains from 8 to 10 cm depth. 5 July 1966. Sample taken at a low-water station.

PLATE 2

Figs. 4–6. Incident ultraviolet illumination with a Carl Zeiss microscope oil immersion N.A. 1·3. Surfaces of sand grains from low water station, stained with acridine orange (1/62,000%, w/v) showing numerous bacteria and also diatoms. 4 September 1966.

PLATE 3

Figs. 7–12. Photographed with a Carl Zeiss Phase microscope, oil immersion N.A. 1·25 unstained. All photographs at same magnification.



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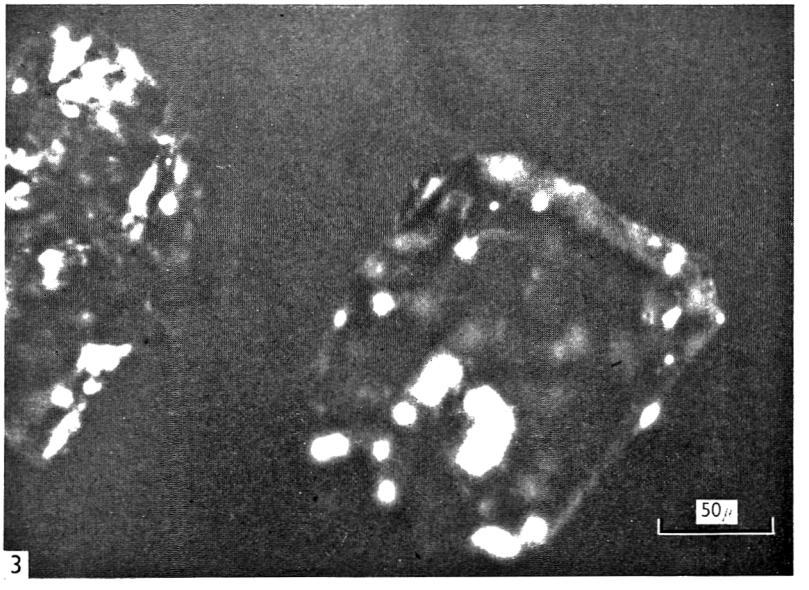

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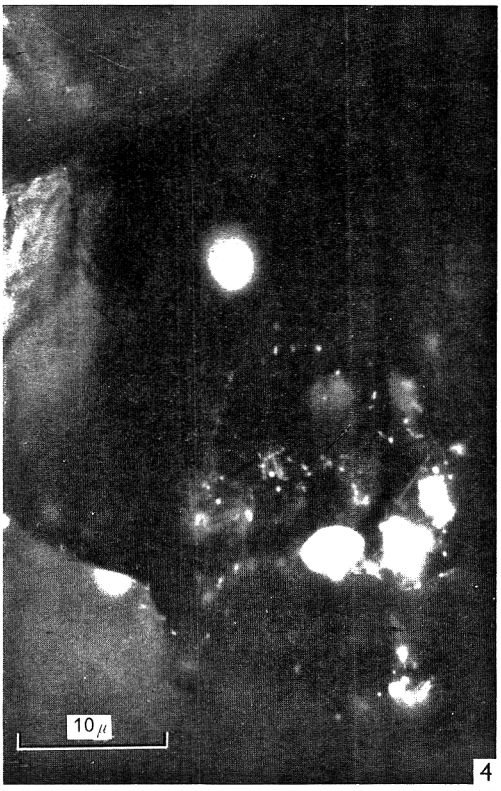
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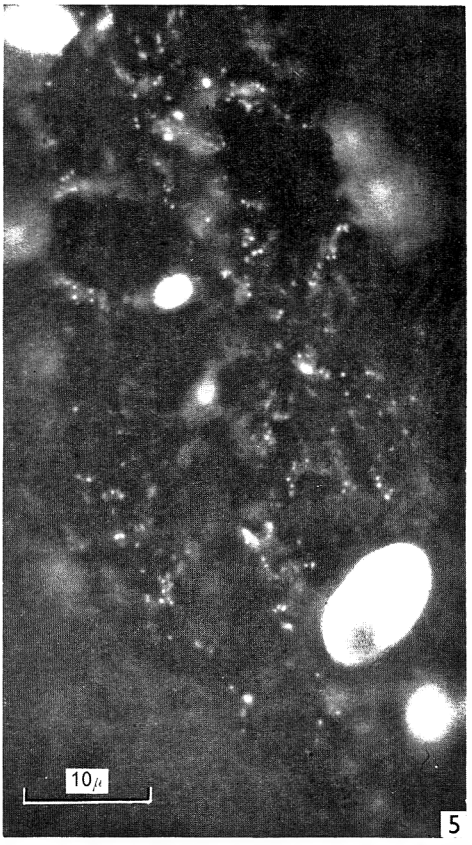
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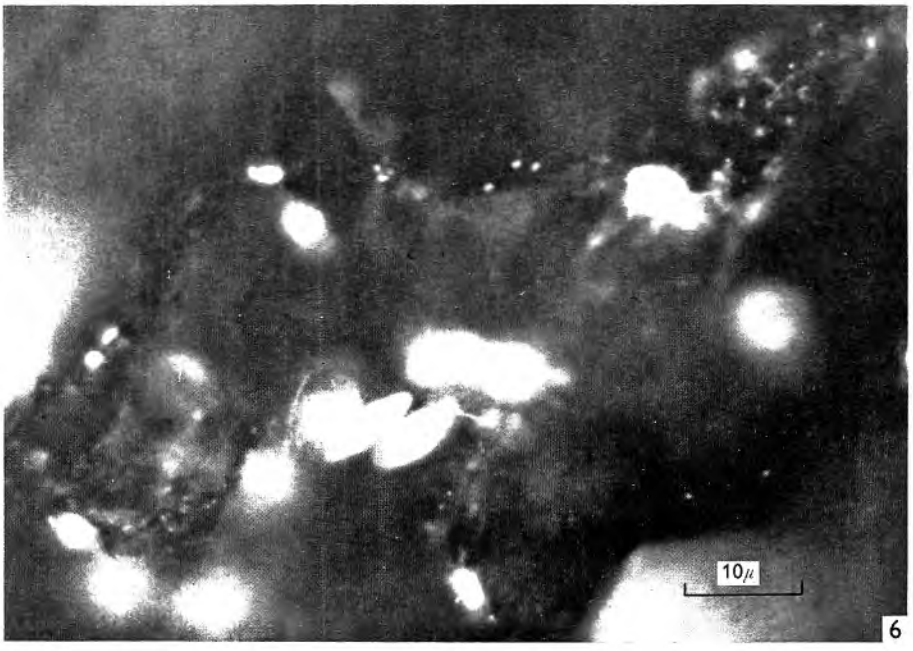
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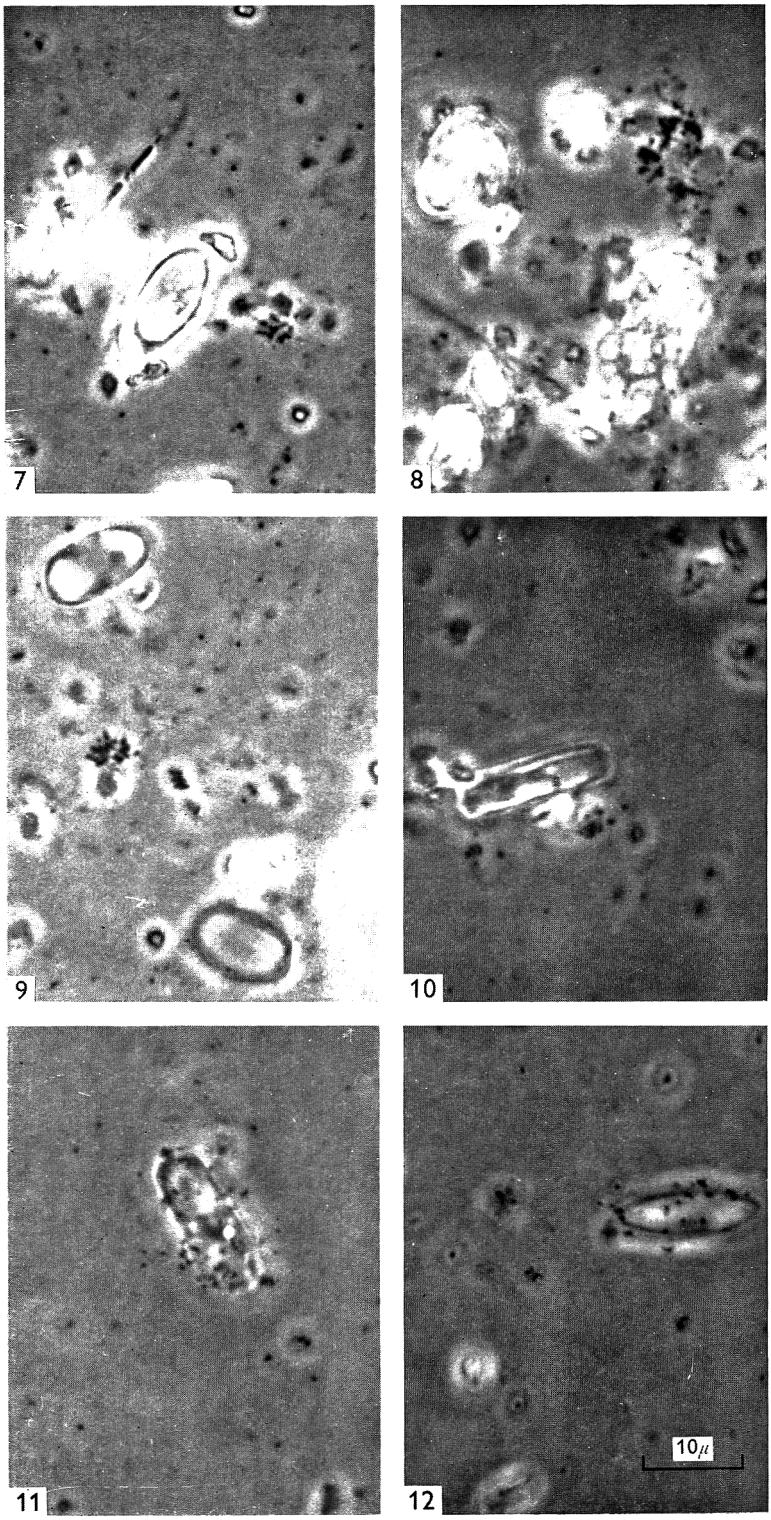
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J. gen. Microbiol. (1968), **51**, 43–48
Printed in Great Britain

Use of Inhibitors for Selective Isolation and Enumeration of Cytophagas from Natural Substrates

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(*Accepted for publication* 4 *September* 1967)

Several antibacterial agents were tested against a range of cytophagas and other bacteria representing types commonly found in soil and marshy places. From these tests two antibiotics (penicillin 15 units/ml. and chloramphenicol 5·0 μg./ml.) and two bile salts (sodium taurocholate 8·0 mg./ml. and sodium cholate 6·0 mg./ml.) were selected for incorporation into alkaline enrichment media to achieve selective growth of cytophagas. This mixture of antibiotics and bile salts was tested with a number of soils and its efficiency compared with several other methods for selective development of cytophagas; bile salts inhibited two common species. The most suitable mixture for the selection of cytophagas was enrichment medium with penicillin and chloramphenicol.

INTRODUCTION

The current methods for primary isolation of soil cytophagas orient around enrichment in liquid media containing cellulose or chitin, followed by streaking on solid media with the same enrichment. The isolation methods recommended by Bachman (1955), Stanier (1942) and Veldkamp (1961) entail several transfers in the elective environment. Although antibiotics have been commonly used in media to select actinomycetes, fungi and other bacteria, they have not been used for selective isolation of cytophagas, with the exception of actidione for the isolation of fruiting myxobacteria (Brockman & Boyd, 1963). However, this antifungal antibiotic has a restricted action against bacteria, which are the usual contaminants. Therefore, it was decided to make a study of the value of antibacterial agents for the selection of cytophagas. The inhibitors were tested in glucose mineral salt solution (Stanier, 1942), mineral chitin agar (Stanier, 1947) and Veldkamp medium (Veldkamp, 1961), commonly used for the study of cytophagas. The effect of antibiotics and bile salts on pure cultures of soil-inhabiting cytophagas was earlier reported (Warke & Dhala, 1966) where penicillin, chloramphenicol and bile salts were incorporated in alkaline enrichment media in several comparative experiments.

METHODS

The cellulolytic, chitinoclastic and facultative anaerobic Cytophaga species (Table 1) earlier isolated by enrichment techniques were maintained, grown and tested on glucose mineral salt solution, mineral chitin agar and Veldkamp's agar, respectively.

Testing of inhibitors against cytophagas and other bacteria. Minimal inhibitory concentrations (m.i.c.) of sodium penicillin G (Pfizer) and chloramphenicol (Pfizer), were determined by the tube dilution method; solutions of the antibiotics were prepared

in sterile water and added to give concentrations of chloramphenicol 5·0–6·5 μg./ml. and Na penicillin G 10, 15, 20 and 25 units/ml. Aqueous solutions of bile salts 10 % (w/v) were sterilized at 121° for 20 min. and added to give 5, 10, 15 and 20 mg./ml. Tests were run in tubes, each containing 5 ml. Pen-assay broth (Difco). The tubes were inoculated to give 2×10^6 organisms/ml. The cultures were inoculated and incubated at 28° for 48 hr, and the degree of growth determined in terms of turbidity measurements with a Klett–Summerson colorimeter at 450 mμ. The inhibitory effect was further confirmed by streaking loopfuls from dilution tubes on to a nutrient agar surface. The reaction of some common soil bacteria which grew in the enrichment medium, e.g. Cellulomonas, Desulphovibrio, Pseudomonas, Bacillus and Nocardia species earlier isolated from enrichment tubes, was tested against the selected concentrations of inhibitors (Table 2); the inhibitors were incorporated in glucose mineral agar for Cellulomonas, Cellvibrio, Desulphovibrio and Pen-assay agar (Difco) for the rest.

Table 1. *Effect of penicillin, chloramphenicol and bile salts on various Cytophaga species*

	Minimal inhibitory concentration			
Cytophaga species	Penicillin (units/ml.)	Chloramphenicol (μg./ml.)	Sodium taurocholate (mg./ml.)	Sodium cholate (mg./ml.)
Cytophaga hutchinsonii	25	6·0	20·4	20·0
C. lutea	15	5·0	15·8	16·0
C. rubra	18	5·0	—	—
C. tenuissima	15	5·5	—	—
C. aurantiaca	15	5·5	6·4	8·0
C. albogilva	20	6·0	15·0	13·3
C. deprimata	20	6·0	16·0	13·0
C. johnsonii	26	5·5	11·0	12·0
C. fermentans	29	6·0	20·0	15·8
C. fermentans var. *agarovorans*	25	6·3	19·5	17·5
C. salmonicolor	27	6·7	19·8	15·75

Comparison of soil enrichment tubes with and without inhibitors. From the results of tests with pure cultures, experiments were done to study the effect of the inhibitors on colony counts on dilution plates prepared from several soil and mud samples. The soils used were: a garden soil from Andheri, Bombay (pH 6·2); soil from cotton field, College campus, Bombay (pH 5·9); manured soil from Bandra, Bombay (pH 6·0); two horizons from Aarey, Bombay, the A horizon being at pH 4·2–5·8 and the B horizon at pH 7·2–8·6; two marshy soils from Versova and Danda, Bombay, at pH 6·7–7·1. These soils differed considerably in their total microbial contents and the relative proportion of cytophagas to other bacteria.

Isolation. Soil (1 g.) was added to 100 ml. sterile water in screw-top bottles and shaken on a reciprocal shaker for 30 min. Tenfold dilutions were prepared from the resulting suspension and 0·1 ml. of 10^{-3} and 10^{-4} dilutions were used to seed 10 ml. of the enrichment medium (Stanier, 1942). The inhibitors when used were added to enrichment medium just before inoculation at the following concentration: Na penicillin G 15 units/ml.; chloramphenicol 5·0 μg./ml.; bile salts 5·0 mg./ml. Experiments were run in 15 sets for each dilution. Tubes were incubated at 28° for 8 days;

slimy patches from the cellulose strips were scraped out with a platinum needle and streaked on corresponding sets of solid media with 0·75 % agar (U.S.P., Japan) and 0·5 % glucose instead of cellulose. Re-streakings on fresh media were often necessary to obtain pure cultures. Besides these media, starch agar and cellulose + dextrin agar (Fuller & Norman, 1942) containing the inhibitors were also used in the isolation of *Cytophaga albogilva* and *C. deprimata*. Chitinoclastic cytophagas were isolated by inoculating 1 g. soil in a flask containing Stanier's basal medium (1947) with and without inhibitors and with chitin strips (prepared from lobster shells; Stanier, 1947) as sole source of carbon. Growth from chitin strips after 8 days of incubation at 28° was streaked on mineral chitin agar. Facultative agar-decomposing cytophagas were enriched according to Veldkamp's technique (1961) with the inhibitory substances incorporated in enrichment medium.

Table 2. *The reaction of some cytophagas and other bacteria to inhibitors*

Inhibitors	Completely inhibited	Partially inhibited	Not inhibited
Penicillin (15 units/ml.) + chloramphenicol (5·0 μg./ml.) (*a*)	Cellulomonas, Nocardia Pseudomonas species	Desulphovibrio and Cellvibrio species, *Bacillus subtilis*	Cytophaga species*
Sodium taurocholate + sodium cholate (5·0 mg./ml., each) (*b*)	*Cytophaga rubra*, *C. tenuissima* Nocardia Bacillus species	Cellulomonas, Desulphovibrio Cellvibrio species	Cytophaga species* except *C. rubra* *C. tenuissima*
a + *b*	*C. rubra*, *C. tenuissima* Nocardia, Pseudomonas, Cellulomonas Bacillus species	Cellfalcicula, Desulphovibrio Cellvibrio species	Cytophaga species* except *C. rubra* *C. tenuissima*

* Cytophaga species which are mentioned in Table 1.

RESULTS

Testing of inhibitors against cytophagas and other bacteria

Of the four inhibitors tested against a range of cytophagas, penicillin (15 units/ml.) and chloramphenicol (5·0 μg./ml.) at pH 9·0 were tolerated by all the tested strains. It is evident from Table 1 that bile salts were well tolerated by the majority of cytophagas, except for *Cytophaga rubra* and *C. tenuissima*, which were highly sensitive to bile salts. It was decided to test the sensitivity of the strains against a mixture of penicillin, chloramphenicol and bile salts in various concentrations at pH 9·0.

The reaction of some bacteria to inhibitors

Details of the reaction of the individual Cytophaga species and some common contaminants to the inhibitory mixtures are given in Table 2. The results were like those previously obtained; only the presence of bile suppressed the growth of some cellulolytic cytophagas.

Comparison of soil enrichment tubes with and without inhibitors

Results obtained with enrichment medium with and without inhibitors are given in Table 3. It is seen that the isolation of cytophagas was facilitated since the ratio of cytophaga to bacteria was increased, whereby the chances of isolation are enhanced. Similarly, incorporation of the antibiotics and bile salts decreased the number of secondary transfers in enrichment media and made it possible to obtain cytophagas directly on the dilution plate. The usefulness of the inhibitory substances was seen with all types of Cytophaga, namely cellulolytic, chitinoclastic and also facultatively anaerobic agar-decomposing forms.

Table 3. *Selective enrichment medium for the isolation of cytophagas*

	Type of cytophagas					
	Cellulolytic		Chitinoclastic		Facultative anaerobes	
Media	C to B* ratio	No. of secondary transfers in EM	C to B ratio	No. of secondary transfers in EM	C to B ratio	No. of secondary transfers in EM
EM†	1:60	3	1:65·5	2	1:70	3
EM+sodium taurocholate (EMT)	1:33	2	1:37	1	1:30·5	1
EM+sodium cholate (EMC)	1:34	2	1:35·2	1	1:29	1
EM+penicillin (EMP)	1:38	2	1:41	1	1:38	2
EM+chloramphenicol (EMCh)	1:30	2	1:32	1	1:28·5	1
EM pH 8·5 (EM 8·5)	1:31	2	1:30·5	1	1:27·3	1
EMT+sodium cholate	1:28	1	1:25·5	Nil	1:23·5	Nil
EMT+penicillin	1:22	1	1:22	Nil	1:20·2	Nil
EMT+chloramphenicol	1:20	Nil	1:21	Nil	1:18·5	Nil
EMC+penicillin	1:18·3	Nil	1:19	Nil	1:16·2	Nil
EMC+chloramphenicol	1:17·2	Nil	1:16	Nil	1:15·3	Nil
EM 8·5+sodium cholate	1:15·3	Nil	1:14	Nil	1:13·2	Nil
EM 8·5+sodium taurocholate	1:14·5	Nil	1:14	Nil	1:13·7	Nil
EM 8·5+chloramphenicol	1:13	Nil	1:13·2	Nil	1:12·5	Nil
EMCh+penicillin	1:12·5	Nil	1:13	Nil	1:11·6	Nil

* C = Cytophagas, B = Bacteria; † EM = Enrichment medium.

Comparison of plate counts obtained on alkaline cytophaga agar medium, unsupplemented and with four inhibitors

Plate counts of Cytophaga colonies were made for seven soil samples on alkaline Cytophaga agar containing penicillin + chloramphenicol + bile salts, the same medium with only antibiotics, and two other media selective for cytophagas. Details of these results are given in Table 4. When the percentages of colonies on plates were considered, the alkaline Cytophaga agar with antibiotics gave results almost similar to those obtained with the medium containing four inhibitors. Unsupplemented alkaline Cytophaga agar medium had only 4—11 % of Cytophaga colonies out of the total colonies developing on plates, indicating a good selectivity of the supplemented media.

Table 4. *Comparison of colony counts obtained on alkaline cytophaga agar, unsupplemented and with inhibitors*

Soil sample	Dilution of soil suspension		With mixture of four inhibitors* colonies** (%)	With anti-biotics† colonies (%)	With bile salts‡ colonies (%)	Without inhibitors§ colonies (%)	Change on plates with: Anti-biotics (%)	Bile salts (%)	Anti-biotics + bile salts (%)
Andheri garden soil	$1/10^4$	Cytophagas	27	25	20	9	+16	+11	+18
		Bacteria	73	75	80	91			
Bandra manure soil	$1/10^4$	Cytophagas	40	33	40	10	+23	+23	+30
		Bacteria	60	67	60	90			
Cotton field soil	$1/10^4$	Cytophagas	20	18	14	7	+11	+ 7	+13
		Bacteria	80	82	86	93			
Fresh field soil, A horizon	$1/10^3$	Cytophagas	22	34	25	10	+24	+15	+12
		Bacteria	78	66	75	90			
Fresh field soil, B horizon	$1/10^3$	Cytophagas	36	26	33	11	+15	+22	+25
		Bacteria	64	74	67	89			
Versova marshy soil	$1/10^2$	Cytophagas	14	9	9	4	+ 5	+ 5	+10
		Bacteria	86	91	91	96			
Danda marshy soil	$1/10^2$	Cytophagas	30	20	19	7	+23	+12	+23
		Bacteria	70	80	81	93			

* Four inhibitors = penicillin (15 units/ml.)+ chloramphenicol (5 μg.ml.)+Na cholate+Na taurocholate 6 mg./ml. each.
** Average number of colonies per plate as %.
† Penicillin (15 units/ml.) +chloramphenicol (5 μg./ml.).
‡ Sodium cholate+sodium taurocholate (6 mg./ml. each).
§ Tested by using cytophaga agar, glucose mineral agar and Veldkamp agar.

DISCUSSION

The proportion of cytophagas to the other micro-organisms in most soil samples is very small; this limits the use of the dilution method for isolations. Besides, the cytophagas exhibit a spreading type of growth and carry along the contaminants, which remain embedded in their slime in several subcultures. The sensitivity of cytophagas to heat above 40° and their loss of pigmentation even at 37° (Warke & Dhala, 1966) rules out attempts to restrict the growth of other bacteria by heat treatment. The common method of isolation of cellulolytic cytophagas depends particularly on the formation of coloured patches on filter-paper strips (Stanier, 1942; Fuller & Norman, 1943); these usually have many non-cellulolytic and non-myxobacterial forms which thrive on breakdown products of cellulose produced by a few cellulolytic varieties. Similarly, the chitin strips underwent a slow decomposition accompanied by the development of a varied microflora, particularly of chitin-decomposing eubacteria, from which cytophagas could not be readily obtained. However, these eubacteria were easily suppressed in selective media where the cytophagas were often observed to be the only chitin decomposers. The facultative anaerobes could likewise be equally well isolated from marine sources. A great advantage of the inhibitors tested was noticed in the isolation of marine forms, where blackening of the enrichment media was prevented since the sulphur-reducing bacteria were also eliminated.

Although Bachmann (1955) reported a failure to develop a more suitable method for enrichment of facultatively anaerobic cytophagas, *Cytophaga fermentans* was readily isolated by the incorporation of penicillin (15 units/ml.) + chloramphenicol (5·0 μg./ml.) in isolation media.

It was noticed that of the seven soil samples (Table 4) examined for Cytophaga species by the dilution method, with and without inhibitors, almost all the samples which yielded cytophagas in absence of the inhibitors also gave the same species in presence of the inhibitors (either singly or mixed) except for bile salts, which were toxic to *Cytophaga tenuissima* and *C. rubra*.

It is felt that the use of inhibitors can be applied profitably in future studies on the isolation of cytophagas from natural sources.

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Printed in Great Britain

Heritable Glycogen-storage Deficiency in Yeast and its Induction by Ultra-violet Light

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(*Accepted for publication 5 September 1967*)

SUMMARY

Cultures of several diploid strains of *Saccharomyces cerevisiae* were found to contain about 0·5 % of glycogen-deficient cells. Ultraviolet irradiation increased the mutant frequency to about 5 %, while 40 % of the population survived. The mutants were detected by plating cultures on nutrient agar containing 1 % glucose and staining 3-day colonies with iodine; normal colonies became brown due to the stored glycogen whereas the mutants remained white. The size of the mutant colonies was normal. Most of the mutants have remained stable during subculture. Fractionation of the cellular carbohydrate confirmed that the mutants were deficient in glycogen. Glucose concentration in the growth medium had a marked influence on glycogen storage. When the mutants were grown on nutrient agar containing 8 % glucose, glycogen was stored and the mutants could not be distinguished by iodine-staining from the wild type. Glycogen-deficient mutants contained higher than usual proportions of respiration-deficient cells. Respiration-deficient colonies, whether derived from glycogen-deficient or from normal cultures contained appreciably less glycogen than those of the wild-type yeast.

INTRODUCTION

Iodine staining has been much used for the examination of polysaccharide storage by bacteria (Carlson & Hehre, 1949; Gibbons, 1964; Carrier & McCleskey, 1962). Yeasts may differ in their ability to store glycogen (Chester, 1964) and the present work on glycogen-deficient mutants arose from an attempt to apply an iodine-staining test to yeast colonies to reveal the storage of glycogen.

METHODS

The strains of *Saccharomyces cerevisiae* used in these experiments were non-sporing diploid Guinness brewing yeasts. Most of the work was done with strain 4236; the other strains used were 1164 and 1338. Strain 1338 is a flocculent yeast.

The media used for growing the yeast had the following compositions:

Medium 1. Malt extract, 3 g.; yeast extract 3 g.; peptone, 5 g.; glucose 10 g.; distilled water to 1 l. (Wickerham, 1951).

Medium 2. This had the same composition as medium 1 except that it contained 80 g. glucose.

Solid media for plating were prepared by adding one agar tablet (Oxoid) to 10 ml. of liquid medium in 1 oz. (28 ml.) screw-top bottles. The Petri dishes had a diameter of 8·5 cm. All cultures were incubated at 24°.

Glycogen gives a reddish-brown coloration with iodine, and glycogen-deficient mutants were detected by flooding 3-day or 6-day colonies on plates of solid medium 1, with 6 ml. iodine solution (0·2 % I_2 in 0·4 % KI). The staining reactions of the colonies were recorded 1 min. after adding the iodine. The reactions of the various types of colonies are described in the text. When colonies were isolated from plates which had been flooded with iodine solution, sufficient viable organisms survived the treatment to give subcultures.

For ultraviolet (u.v.) irradiation, yeast cells were harvested from 24 hr cultures in medium 1, washed once with distilled water and suspended in distilled water. Four-ml. portions of the suspension (about 2×10^7 cells/ml.) were u.v.-irradiated at room temperature (19°) in Petri dishes. The u.v. source was a Hanovia Chromatolite u.v. lamp at a distance of 16 cm. from the cell suspension, which was gently agitated during irradiation.

Spontaneous respiration-deficient mutants were obtained by plating cultures and staining the colonies with triphenyltetrazolium chloride (Ogur, St John & Nagai, 1957). Normal colonies were stained pink and respiration-deficient colonies remained white. This method was also used to test the glycogen-deficient mutants for respiration deficiency.

Yeast carbohydrate was fractionated by the scheme described by Trevelyan & Harrison (1956).

RESULTS

Iodine-staining of yeast colonies

Several strains of yeast were plated on nutrient agar (medium 1) and the plates flooded with iodine solution after incubation for 3 days. The colonies from all the cultures were stained brown because of the glycogen stored in the cells. There was little change in coloration when the colonies were incubated for 6 days before staining.

Frequency of spontaneous glycogen-deficient mutants

On plates of strains 1164, 1338 and 4236 there were some colonies which remained white or very pale yellow in the presence of the iodine. When these unstained colonies were picked off, grown in medium 1 and replated, all the progeny gave a negative reaction with iodine. The frequency of the aberrant colonies was as high as 0·8 % with some strains (Table 1). When normal colonies of strain 4236 were picked off the plates, grown overnight in medium 1 and replated, a similar high frequency of glycogen-deficient colonies was observed. In liquid medium, the growth and rate of fermentation of the glycogen-deficient mutant of strain 4236 were slightly slower than those of the parent yeast. The differences, although very small, were consistent in many experiments. Thus it seems unlikely that the high frequency of the mutants was caused by a selective advantage in growth rate. This conclusion is supported by the fact that there was the same high frequency of mutants in the freshly isolated strains as in the stock cultures which have been maintained by subculture for several years.

Induction of glycogen-deficient mutants with ultraviolet radiation

Washed suspensions of strain 4236 were u.v.-irradiated for 15–60 sec. The treated suspensions were diluted with sterile water and plated on solid medium 1. Iodine-staining of the colonies showed that the frequency of the glycogen-deficient mutants

was increased by the u.v.-irradiation. After 30 sec. irradiation the frequency had increased sevenfold while over 60 % of the population survived the treatment. A 45 sec. dose increased the frequency about tenfold while 40 % of the population survived (Fig. 1). These figures show that the effect was actually an induction and not a differential killing of normal and mutant cells. The mutant frequency tended to decrease after longer u.v. treatment.

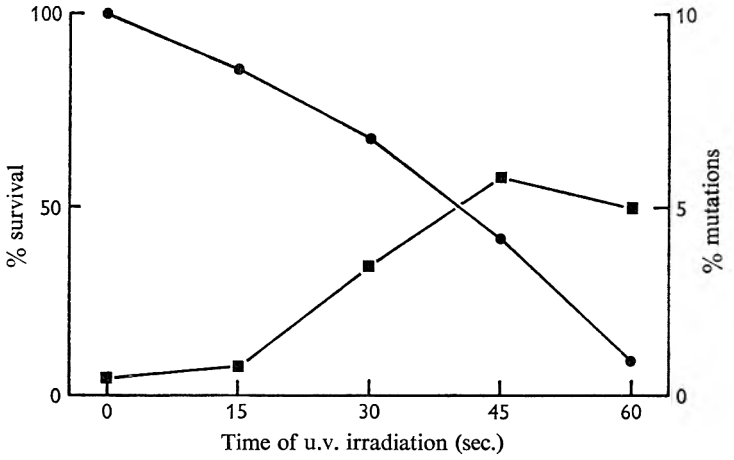

Fig. 1. Ultraviolet dose/effect curves for *Saccharomyces cerevisiae* strain 4236.
% Survival (●——●), % mutations(■——■).

Table 1. *Frequency of glycogen-deficient mutants of Saccharomyces cerevisiae*

Strain	Total no. of colonies	Glycogen-deficient colonies	Frequency (%)
1164	1220	6	0·5
1338	1304	10	0·8
4236	1368	8	0·6
4236/1*	2298	10	0·4
4236/2	2812	1	< 0·1
4236/3	1139	5	0·4
4236/4	1812	2	0·1
4236/5	1223	5	0·4
4236/6	1366	3	0·2

* 4236/1–4236/6 were fresh isolates from culture no. 4236.

In the experiments with u.v. radiation, many of the colonies were sectored and these were counted as mutants. Ten of the sectored colonies were isolated from the plates, grown overnight in medium 1 and replated. As expected, iodine-staining showed that the plates were mixtures of normal colonies and glycogen-deficient colonies.

Ultraviolet radiation is known to induce respiration deficiency (Raut, 1954). None of the ten glycogen-deficient isolates examined in this experiment was respiration-deficient.

Stability of glycogen-deficient mutants

Glycogen-deficient mutants were maintained by subculture at monthly intervals on nutrient agar slopes. Nine isolates of spontaneous mutants remained stable for over a

year, but three others were found to contain a proportion of reverted organisms. Ultraviolet-induced mutants were maintained for a shorter period, but have all remained stable.

Effect of glucose concentration in the growth medium on iodine-staining

The glucose concentration in the solid growth medium influenced the glycogen content of the cells. On medium 2 (8% glucose) glycogen-deficient mutants were stained by iodine as much as were the wild-type yeasts (Table 2).

Table 2. *Saccharomyces cerevisiae: iodine-staining of colonies*

	Grown on			
	Medium 1		Medium 2	
	3-day	6-day	3-day	6-day
Strain	Colour on treatment with iodine			
4236	Brown	Brown	Brown	Brown
Glycogen-deficient 4236	White	White/ pale brown	Brown	Brown
Respiration-deficient 4236	Brown	White*	Brown	Pale brown/ white

* The less crowded colonies on these plates were white at the centre and brown around the circumference.

Respiration-deficient mutants

Various types of yeast, especially species of *Saccharomyces*, contain a proportion of respiration-deficient (r.d.) cells (see review by Ephrussi & Hottinguer, 1951). These r.d. cells are slow-growing and give very small colonies, usually referred to as 'petites'; petite colonies usually appear with a frequency of less than 1%. Colonies of the glycogen-deficient mutants were not obviously different in size from wild-type colonies and were stained pink by triphenyltetrazolium chloride, indicating that they were not respiration-deficient. However, most cultures of the glycogen-deficient mutants contained higher than usual proportions of r.d. cells. Cultures of glycogen-deficient mutants isolated from strain 4236 contained 2–9% of r.d. cells, whereas the wild type contained about 0·5%. In contrast to the glycogen-deficient mutants, iodine solution stained 3-day petite colonies brown, but more mature 6-day colonies remained white (Table 2).

An unexpected observation was that spontaneous r.d. mutants isolated from normal yeast cultures gave iodine-staining reactions similar to those of the r.d. mutants isolated from glycogen-deficient cultures. The very small 3-day colonies growing on medium 1 were stained brown with iodine; 6-day colonies remained white with iodine solution although the less crowded colonies on the plates were stained brown around the circumference. When grown on medium 2, all the r.d. colonies were larger than when grown on medium 1. There was less distinction between 3-day and 6-day plates on the high-glucose medium, but in all strains there was a decrease in intensity of staining as the colonies grew older and larger.

Carbohydrate fractionation of yeasts grown on agar growth medium

Strain 4236 and a glycogen-deficient mutant isolated from it were grown on agar slopes of medium 1 and after incubation for 3 days at 24° samples of the yeast were washed 3 times with distilled water and the carbohydrate of the cells fractionated. The carbohydrate fractions are expressed as a percentage of the total dry weight of the yeast and also as a percentage of the non-carbohydrate dry weight. Under the present growth conditions, the glycogen contributes from 8 to 17% of the total dry weight. The variations in glycogen content influence the numerical value of the other carbohydrate fractions when they are expressed as a fraction of total dry weight. Expression of the results as a percentage of non-carbohydrate dry weight overcomes this difficulty and facilitates a comparison of the two yeasts.

Table 3. *Saccharomyces cerevisiae: fractionation of cellular carbohydrate of strain 4236 and a glycogen-deficient mutant*

The yeasts were harvested from the agar after incubation (24°) for 3 days. Results are expressed as (1) % of yeast dry wt, (2) % of yeast non-carbohydrate dry wt (dry wt *minus* total carbohydrate).

	Strain 4236		Glycogen-deficient mutant	
	(1)	(2)	(1)	(2)
Trehalose	0·6	0·9	0·2	0·3
Alkali-soluble glycogen	1·7	2·4	1·7	2·2
Acid-soluble glycogen	16·0	22·7	7·1	9·1
Mannan	5·6	8·0	6·7	8·6
Glucan	5·7	8·1	6·0	7·7
Sum of fractions	29·6	42·1	21·7	27·9

Table 4. *Saccharomyces: cerevisiae fractionation of cellular carbohydrate of a respiration-deficient mutant of strain 4236*

Results are expressed as (1) % of yeast dry wt, (2) % of yeast non-carbohydrate dry wt (dry wt *minus* total carbohydrate)

	3-day culture		6-day culture	
	(1)	(2)	(1)	(2)
Trehalose	0·5	0·7	0·2	0·3
Alkali-soluble glycogen	0·9	1·2	1·4	1·8
Acid-soluble glycogen	9·8	13·0	6·8	8·8
Mannan	8·9	11·9	8·4	10·9
Glucan	4·8	6·4	6·3	8·2
Sum of fractions	24·9	33·2	23·1	30·0

The mutant contained less than half as much acid-soluble glycogen as the wild type and about one third as much trehalose (Table 3). The alkali-soluble glycogen was the same in both yeasts. There was also little difference in the cell-wall components, glucan and mannan. Similar analyses were made on 3-day and 6-day cultures of an r.d. mutant of strain 4236. The 6-day cultures contained less glycogen and trehalose than the younger cultures (Table 4). Having regard to the decrease in intensity of iodine-staining as the r.d. cultures aged, this result was to be expected. The 3-day culture of the r.d. yeast contained appreciably less glycogen than the parent cultures of the same age.

Respiration-deficient mutants derived from glycogen-deficient mutants could not be distinguished by iodine-staining from the r.d. mutants derived from normal cultures. Fractionation of the cellular carbohydrate of an r.d. mutant derived from a glycogen-deficient culture confirmed that the glycogen content was similar in the two cases (Tables 4 and 5).

Table 5. *Saccharomyces cerevisiae: fractionation of cellular carbohydrate of a respiration-deficient mutant derived from a glycogen-deficient mutant of strain 4236*

Results are expressed as (1) % of yeast dry wt, (2) % of yeast non-carbohydrate dry wt (dry wt *minus* total carbohydrate)

	3-day culture		6-day culture	
	(1)	(2)	(1)	(2)
Trehalose	0·5	0·7	0·2	0·3
Alkali-soluble glycogen	1·1	1·5	1·2	1·6
Acid-soluble glycogen	11·5	14·8	7·1	9·3
Mannan	7·9	10·9	8·1	10·5
Glucan	6·2	8·5	6·4	8·3
Sum of fractions	27·2	36·4	23·0	30·0

DISCUSSION

Spontaneous heritable changes in a particular character tend to be infrequent and there are few examples in the literature of u.v.-radiation-induced mutants as high as 5% of the surviving cells. James (1954) induced a change to galactose-negative of about 10% in a haploid strain of *Saccharomyces cerevisiae* with u.v.-radiation; spontaneous mutants did not exceed 0·5%. Genetic analysis indicated that the negative character had a nuclear origin. In the present work the cultures used were diploid, which makes the high frequency of the spontaneous and u.v.-induced mutants even more surprising. It is hoped to extend this work in an attempt to find spore-forming strains in which glycogen-deficient mutants are as distinctive as those of the asporogenous strains. Genetic analysis should then indicate whether the glycogen-deficiency is due to nuclear or cytoplasmic changes.

Fractionation of the cellular carbohydrate showed that the mutants contained considerably less glycogen than did the wild-type yeast. Despite the fact that the fractionation procedure of Trevelyan & Harrison (1956) indicated that the mutants did contain some glycogen, the colonies remained white in the presence of iodine. It would therefore appear that the iodine test requires a certain concentration of glycogen to be present in the cells before a colour becomes evident. An alternative possibility is that all the material which appears as glycogen in the fractionation procedure may not be typical glycogen as regards iodine-staining.

The mutants were only detectable when grown on the low glucose medium. A high glucose concentration resulted in greater glycogen storage by the mutant and the iodine stain could not differentiate mutant from wild-type colonies. An alternative pathway of glycogen synthesis may become operative when the glucose concentration is above a certain value.

The trehalose as well as the glycogen content was low in the mutant, suggesting that the mutation results in changes in a common synthetic pathway or metabolite. Uridine diphosphoglucose is involved in the synthesis of both carbohydrates and unpublished

results have shown that the concentrations of this metabolite are lower in the mutant cells.

During this work on glycogen-deficient yeasts it was necessary to examine the glycogen storage of r.d. yeast because of possible difficulties in diagnosis of the two types. However, in the strains of yeast which have so far been examined there is no possibility of confusion, because glycogen-deficient colonies are scored after 3 days of incubation, when r.d. mutants stain brown with iodine (Table 2). In addition to the iodine-staining reaction, the r.d. colonies are much smaller than those of the glycogen-deficient mutants.

Although the 3-day r.d. colonies stain with iodine, their glycogen content is low (Table 4), and as the colonies age the content decreases, probably as a result of the glycogen being metabolized as the supply of glucose diminishes. The low glycogen content of r.d. cells in colonies on agar plates is of some theoretical interest because when the cells were grown in liquid culture they accumulated similar quantities of carbohydrate to those of the parent yeasts (Chester, 1963). A possible explanation for the low glycogen content of the r.d. mutants is as follows. Adenosine triphosphate (ATP) is required in the cell for at least two reactions in the synthetic pathway to glycogen: it is necessary for the hexokinase reaction in which glucose is phosphorylated, and also for the conversion of uridine diphosphate to uridine triphosphate (Berg & Joklik, 1953). Uridine triphosphate reacts with glucose-1-phosphate to form uridine diphosphoglucose (Trucco, 1951). Glycogen is formed by transfer of glucose from uridine diphosphoglucose to a primer (Leloir & Cardini, 1957). The glucose concentration inside a yeast colony is dependent on diffusion from the nutrient agar. As the glucose is depleted by yeast growth the amount diffusing into the colony may be insufficient for all the needs of the cell, especially in the larger colonies on the 6-day plates. A low glucose concentration around the cells, combined with the necessity for r.d. cells to obtain their energy by fermentation instead of the more efficient process of respiration, may result in the cells having insufficient ATP for glycogen synthesis. The less crowded colonies on 6-day plates were stained brown with iodine around the circumference (Table 2); this fact lends support to the suggestion that diffusion of glucose into the r.d. yeast colony can be a limiting factor in the storage of glycogen.

The 3-day colonies of the r.d. mutant derived from a glycogen-deficient culture were stained brown by iodine and contained approximately the same amount of glycogen as the r.d. mutant derived from strain no. 4236 (Tables 4 and 5). Assuming that the lesion causing glycogen deficiency was inherited by the r.d. cells, it would appear that the glycogen-deficiency mutation has no effect under conditions of respiration deficiency.

I thank Drs A. K. Mills, C. E. Dalgliesh and R. B. Gilliland for helpful discussion.

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J. gen. Microbiol. (1968), **51**, 57–65
Printed in Great Britain

Regulation of Synthesis of Glutamine Synthetase in *Escherichia coli*

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(*Accepted for publication* 6 *September* 1967)

SUMMARY

The synthesis of glutamine synthetase in *Escherichia coli* under different conditions was studied. The optimal concentration of ammonium sulphate in the growth medium for the enzyme synthesis was 0·4 mM. When glucose was limiting growth the enzyme synthesis became dependent on oxygen. L-Glutamine repressed synthesis of the enzyme and, to a lesser extent, that of glutamate dehydrogenase. L-Arginine, L-asparagine, D-glutamine, L-methionine and L-tryptophan also decreased synthesis of glutamine synthetase. On the other hand, L-glutamate induced the enzyme synthesis; L-isoleucine, L-leucine and L-valine also increased it. Acetyl-L-glutamine did not affect the enzyme synthesis. D-Glutamine was much less active than L-glutamine as a substrate in the γ-glutamyl transfer reaction. At low concentrations, both aza-L-serine and 6-diazo-5-oxo-L-norleucine increased the specific activity of glutamine synthetase in the bacteria.

INTRODUCTION

Glutamine occupies a central position in many metabolic pathways; thus, in microorganisms, the amide nitrogen of glutamine serves, among others, as the nitrogen source for carbamyl phosphate (Pierard & Wiame, 1964), nicotinamide-adenine dinucleotide (Preiss & Handler, 1958), histidine (Neidle & Waelsch, 1959), *p*-aminobenzoic acid (Srinivasan & Weiss, 1961) and glucosamine 6-phosphate (Ghosh, Blumenthal, Davidson & Roseman, 1960). Therefore a control of the synthesis of glutamine might indirectly influence the synthesis and metabolism of a wide variety of compounds. The present study represents an effort to understand how the chemical environment of *Escherichia coli* strain W organisms affects the formation of glutamine synthetase, the enzyme which synthesizes glutamine from glutamic acid, ammonia and adenosine triphosphate.

METHODS

Growth of the bacteria. Escherichia coli strain W (American Type Culture Collection 9637) was grown in a nutrient broth medium (Difco Laboratories) until the logarithmic phase of growth had been reached. One ml. of the broth culture served for inoculating 100 ml. of a defined basal medium which had the same composition as the one used by Davis & Mingioli (1950), except that ammonium sulphate was decreased from 1 to 0·05 g./l. After the culture had been incubated at 37° for 7 hr, the bacteria were harvested by centrifugation and washed once with saline. They were suspended in about

5 ml. of saline, so that the suspension gave a reading between 0·480 and 0·560 at 650 mμ in a Coleman Junior spectrophotometer. Five drops of the bacterial suspension were pipetted into each of several 500 ml. conical flasks containing 200 ml. of the medium under study. Unless stated otherwise, the incubation was at 37° for 16 hr with the flasks undisturbed. At the end of the incubation period, the amount of growth was determined by measuring the turbidity of the culture at 650 mμ in the Coleman Junior spectrophotometer. In the following tables growth is always expressed in terms of absorbance readings with this instrument; a reading of 0·1 corresponded to 0·07 mg. dry bacteria/ml.

Preparation of cell-free extracts. The culture was chilled to 5°. From this point on, all operations were done at 5°. The bacteria were harvested by centrifugation at 10,000 *g* for 10 min. in a Servall SS-3 centrifuge. They were suspended in saline, centrifuged down, and finally suspended in 5 ml. of 0·01 M-tris buffer, (pH 8·2). The bacteria were disrupted by ultrasonic treatment (10 kc., Raytheon Mfg. Co., Waltham, Mass.) for 10 min. The ultrasonically treated preparation was centrifuged at 17,000 *g* for 10 min. The supernatant fluid served for all determinations reported in this study.

Enzyme assays. Glutamine synthetase activity was assayed as described previously (Wu, 1964*a*), except that the reaction mixture was adjusted from pH 7·2 to pH 8·2, the latter being the optimal pH value for the bacterial enzyme. One unit of the enzyme activity is defined as the amount of enzyme that will cause the formation of 1 μmole γ-glutamylhydroxamate in 1 hr at 37°. This assay procedure was used in all determinations for the synthetase activity reported in the following tables and figures. γ-Glutamyltransferase activity was assayed as before (Wu, 1964*b*), but with the following modifications. The reaction mixture contained, per 4·5 ml., 250 μmoles glutamine, 40 μmoles NH_2OH (adjusted to pH 7·2), 10 μmoles $MnCl_2$, 5 μmoles adenosine diphosphate and 45 μmoles phosphate buffer (pH 7·2). The expression for the transferase activity follows that for the synthetase activity. Glutamate dehydrogenase activity was determined according to the procedure of Kennan & Cohen (1961), except that dihydronicotinamide adenine dinucleotide phosphate ($NADPH_2$) replaced dihydronicotinamide adenine dinucleotide ($NADH_2$). The dehydrogenase activity is expressed as μmoles $NADPH_2$ oxidized/mg. protein/min. at 25°. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin as a standard.

Chemicals. All chemicals were of the highest grade available commercially. Most amino acids were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio); acetyl-L-glutamine was obtained from Mann Research Laboratories (New York) and D-glutamine from Calbiochem (Los Angeles, California). Both aza-L-serine and 6-diazo-5-oxo-L-norleucine (DON) were gifts of Dr J. R. Dice (Parke, Davis and Company, Ann Arbor, Michigan). Because of their instability to heat, solutions of these compounds were sterilized by filtration through Millipore filters. Glucose solutions were autoclaved separately and added to the medium later.

RESULTS

The present study will describe first some observations on glutamine synthetase in the cell-free extract of *Escherichia coli*; the induction and repression of this enzyme in the bacteria by compounds of low molecular weight will be considered next.

We found a gradual but continuing increase in glutamine synthetase activity in the cell-free extract when it was stored at $-20°$ (Fig. 1). The increase was largest on the first day, but it continued throughout the period of observation. At the end of 17 days of storage, the enzyme activity had doubled. In these experiments the extract was stored in vials, and one vial was thawed each time for the enzyme assay. We have not explored further the nature of this increase. However, Mecke, Wulff, Leiss & Holzer (1966) identified in crude extracts of *Escherichia coli* a protein that inactivated glutamine synthetase in the presence of adenosine triphosphate $-Mg^{2+}+$glutamine; glutamate could replace glutamine (Mecke, Wulff & Holzer, 1966). Hence, the increase in glutamine synthetase activity observed in the present work (Fig. 1) might result from the spontaneous deterioration of this inactivating protein on storage. On the other hand, it is also possible that activation of glutamine synthetase by an unknown mechanism during storage might account for the increase in its activity. To ensure a uniform basis for comparing the activity data, the enzyme was assayed on the same day the cell-free extracts were prepared. In addition, each experiment was repeated more than once and the results averaged.

The bivalent cation requirement of the bacterial glutamine synthetase was like that of mammalian enzymes (Wu, 1964*b*; Schnackerz & Jaenicke, 1966), in that Mg^{2+} proved to be the most effective activator in the synthesis reaction, and under the same conditions Mn^{2+} lacked activity. On the other hand, Mn^{2+} was required for the transfer reaction. Woolfolk, Shapiro & Stadtman (1966) showed that, at lower pH values than those optimal with Mg^{2+}, Mn^{2+} slightly activated the *Escherichia coli* enzyme in the synthesis reaction. The mechanism underlying the bivalent cation requirement for this enzyme remains to be elucidated.

Like the enzyme from rat liver (Wu, 1964*b*), glutamine synthetase in the cell-free extract of *Escherichia coli* also showed a partial requirement for a sulfhydryl compound for optimal activity. The activity without added sulfhydryl compound averaged 70% of that with the addition. Cysteine (10 mM) or dithiothreitol (2 mM) fulfilled this requirement; 2-mercaptoethanol was less effective.

Woolfolk *et al.* (1966) and Mecke & Holzer (1966) observed that the synthesis of glutamine synthetase in *Escherichia coli* was affected by the ammonium sulphate concentration in the growth medium. We studied this phenomenon in greater detail. We found that higher concentrations of ammonium sulphate supported better growth, but the specific activity of the enzyme in these bacteria was greatly decreased, Fig. 2 shows the results. The highest specific activity was obtained in bacteria grown in the presence of 0·005% (w/v) ammonium sulphate, although growth under these conditions was poor. There was a sharp decline in the specific activity when this concentration of ammonium sulphate was merely doubled. Therefore, in all subsequent experiments in this study, except where stated otherwise, the concentration of ammonium sulphate in the growth medium was 0·005% (w/v; 0·38 mM).

Oxygen also affected the synthesis of glutamine synthetase in *Escherichia coli*, and this effect depended on the glucose concentration. The experiments were made with the flasks containing the inoculated medium either kept stationary, shaken on a rotary shaker, or aerated by bubbling air through a dispersion tube throughout the incubation period. Table 1 summarizes the results. The difference between shaking and aeration was slight and seems insignificant. With a given concentration of glucose, better growth was always obtained in shaken or aerated cultures than with stationary

conditions. However, shaking or aeration increased the specific activity of the enzyme only when glucose concentrations were low, e.g. at 0·5 and 0·25 mg./ml. At higher concentrations of glucose, oxygen no longer limited the enzyme synthesis. The interrelationship between glucose and oxygen would indicate that synthesis of the enzyme proceeds most efficiently only in fully respiring bacteria.

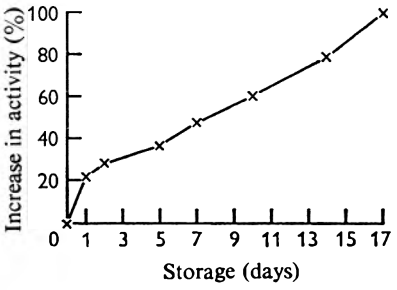

Fig. 1

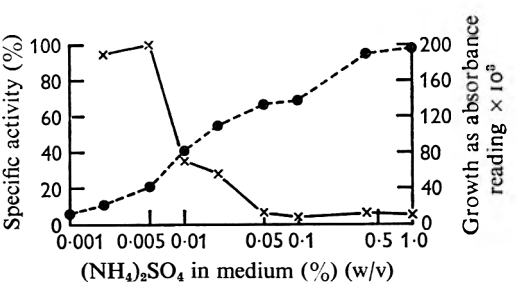

Fig. 2

Fig. 1. Increase in glutamine synthetase activity in cell-free extracts of *Escherichia coli* strain W (ATCC 9637) during storage at −20° C.

Fig. 2. Dependence of growth and synthesis of glutamine synthetase in *Escherichia coli* strain W (ATCC 9637). on the concentration of ammonium sulphate in the basal medium. The specific activity of the enzyme is shown as a % of the value obtained with 0·005% $(NH_4)_2SO_4$. Absorbance, ● - - - - ●; enzyme activity, × —— ×.

Table 1. *Effect of glucose and oxygen on the synthesis of glutamine synthetase in Escherichia coli strain W (ATCC 9637)*

The bacteria were grown in a defined medium with the concentration of glucose varied. During incubation, the medium was either left undisturbed (stationary), shaken, or aerated. The specific activity of the enzyme from the bacteria grown under stationary conditions with 2 mg. glucose/ml. averaged 16·8 units/mg. protein.

	Conditions of incubation					
	Stationary		Shaken		Aerated	
Glucose (mg./ml.)	Cell growth*	Relative specific activity (%)	Cell growth	Relative specific activity (%)	Cell growth	Relative specific activity (%)
0·25	0·027	14	0·042	57	0·055	52
0·5	0·042	33	0·051	135	0·059	161
2	0·046	(100)	0·056	95	0·061	108
4	0·042	110	0·064	103	0·068	129

* Absorbance reading

The repression of an enzyme synthesis by an end product of a reaction catalysed by that enzyme has been amply demonstrated in micro-organisms. Therefore it was not surprising to find repression of glutamine synthetase by glutamine in *Escherichia coli*, as shown in Table 2. The addition of glutamine also resulted in increased growth like that observed with ammonium sulphate (Fig. 2), and the concomitant decrease in specific activity approximated to that obtained with an equivalent amount of ammonium sulphate. The results in Table 2 show that glutamine also repressed, though to a

lesser extent, the synthesis of glutamate dehydrogenase. This observation indicates that the reductive amination of α-ketoglutarate to glutamate was in part utilized for glutamine synthesis. However, since *Escherichia coli* is known to have appreciable glutaminase activity, the possibility that glutamate, a hydrolytic product of glutamine, actually served as a repressor of glutamate dehydrogenase cannot be excluded.

Table 2. *Repression of glutamate dehydrogenase and glutamine synthetase by glutamine in Escherichia coli strain W (ATCC 9637).*

The bacteria were grown in basal medium with glutamine added. In these experiments, the assay of glutamine synthetase was done at pH 7·2.

L-Glutamine (mM)	Glutamate dehydrogenase (μmoles/mg. protein/min.)	Glutamine synthetase (mμmoles/mg. protein/min.)
0	0·57	140
3	0·42	39·4
10	0·32	18·7
30	0·26	7·3
100	0·17	4·0

Table 3. *Effect of various amino acids on the synthesis of glutamine synthetase by Escherichia coli strain W (ATCC 9637)*

In addition to 0·005% of ammonium sulphate, each amino acid was added to the basal medium to a final concentration of 10 mM. The bacteria were harvested after 16 h of incubation at 37°. The bacteria grown in the medium without amino acid addition had a specific activity of glutamine synthetase of 12·7 units/mg. protein for glutamine synthetase.

Amino acid	Amount of growth (absorbance reading)	Relative specific activity of the enzyme (%)
None	0·042	(100)
L-Arginine	0·133	22
L-Asparagine	0·178	16
L-Glutamic acid	0·147	181
L-Glutamine	0·209	9
D-Glutamine	0·172	63
Acetyl-L-glutamine	0·050	107
L-Isoleucine	0·065	120
L-Leucine	0·049	148
L-Methionine	0·094	14
L-Proline	0·041	96
L-Tryptophan	0·086	44
L-Valine	0·061	123

Table 3 shows the induction and repression of glutamine synthetase by other amino acids. We tested two analogues of glutamine, *N*-acetyl-L-glutamine and D-glutamine. Acetyl-L-glutamine slightly stimulated growth but exerted no effect on the enzyme synthesis. The acetyl group appears to have prevented the compound from either entering the cell as freely as glutamine or acting as a repressor of glutamine synthetase. D-Glutamine supported growth almost as well as did L-glutamine, but it repressed the enzyme synthesis to a much lesser extent than did L-glutamine. Glutamic acid was the

only amino acid tested which nearly doubled the specific activity of the enzyme and tripled the bacterial population. Hence, the amount of the enzyme synthesized in the culture with glutamate was about six times that without the amino acid; this amount was further increased by increasing the concentration of glutamate above 10 mM.

Other amino acids showed varied effects on the synthesis of glutamine synthetase. The three branched-chain amino acids, isoleucine, leucine and valine, increased significantly the specific activity of the enzyme in the bacteria, although there was no evidence that they were converted to glutamic acid. But arginine, asparagine, and methionine decreased the enzyme synthesis. Generally speaking, the amino acids which stimulated growth decreased the enzyme synthesis, and vice versa. In other words, the synthesis of the enzyme lagged far behind the synthesis of cellular proteins. Therefore, as growth proceeded, the specific activity of the enzyme diminished.

Since the bacteria could fully utilize D-glutamine for growth with only a slight decrease in the enzyme synthesis, we became interested in learning whether D-glutamine was active as a substrate of the enzyme. The γ-glutamyltransferase activity of the enzyme, which normally requires L-glutamine as a substrate, was therefore assayed. We found that this transferase activity in the extract of bacteria grown in the absence of any added amino acid amounted to 50·8 units/mg. protein with L-glutamine as a substrate, but it approached only 9·2 units/mg. protein with D-glutamine. Hence, D-glutamine had 18% of the activity of L-glutamine. This low activity of the D-isomer for the enzyme may be related to the small repression of enzyme synthesis by this compound.

Table 4. *Effectiveness of amino acids as the sole source of nitrogen for the synthesis of glutamine synthetase by Escherichia coli strain W (ATCC 9637)*

Each amino acid replaced ammonium sulphate in the basal medium and supplied the same amount of nitrogen as that of 0·005% (0·38 mM) of ammonium sulphate. The specific activity of glutamine synthetase in the bacteria grown in the medium with ammonium sulphate averaged 14·0 units/mg. protein in these experiments.

Compound	Incubation time (hr)	Cell growth (absorbance reading)	Relative specific activity of the enzyme (%)
Ammonium sulphate	16	0·041	(100)
L-Arginine	22	0·050	50
L-Asparagine	16	0·074	45
L-Glutamic acid	36	0·055	105
L-Glutamine	22	0·024	96
L-Leucine	96	0·033	137
L-Methionine	96	0·031	63
L-Proline	96	0·022	54

In the experiments just described, a relatively high concentration of an amino acid, 10 mM, supplemented the optimal concentration of ammonion sulphate, 0·38 mM, in the basal medium for the enzyme synthesis. In the following experiments, the ammonium sulphate was replaced by an amino acid solution containing 0·76 milliequivalent of nitrogen per litre. As can be seen in Table 4, only asparagine supported better growth than did the inorganic salt. Growth with each of the other amino acids showed an extended period of the lag phase, which, in certain instances, might have resulted from a low permeability of the cell membrane to the amino acids, such as glutamic

acid. Although asparagine stimulated growth, it decreased synthesis of glutamine synthetase. On the other hand, glutamine appeared to be inferior to asparagine for growth, but it did not repress the enzyme synthesis. Conceivably, the optimal concentration of one amino acid for the enzyme synthesis may differ from that of another amino acid.

The compounds aza-L-serine and 6-diazo-3-oxo-L-norleucine (DON) have been shown to be specific antagonists of glutamine (Levenberg, Melnick & Buchanan, 1957). They have also been shown to inhibit growth of *Escherichia coli* (Kaplan, Reilly & Stock, 1959; Maxwell & Nickel, 1957). These findings suggested that, if concentrations of the compounds were so low in the basal medium that no inhibition of growth

Table 5. *Effect of azaserine and 6-diazo-5-oxo-L-norleucine on the growth and synthesis of glutamine synthetase in Escherichia coli strain W (ATCC 9637)*

Compound (mμg./ml.)		Amount of growth (absorbance reading)	Relative specific activity of the enzyme (%)
Azaserine	0	0·046	(100)*
	5	0·040	113
	10	0·039	117
	20	0·038	133
	40	0·037	121
	50	0·040	90
	100	0·020	63
DON	0	0·043	(100)*
	0·5	0·048	107
	1	0·052	126
	2·5	0·060	102
	5	0·054	44
	10	0·030	26

* This percentage corresponds to a specific activity of 16·8 units/mg. protein.

resulted, the antagonism to glutamine might then induce the bacteria to synthesize more glutamine, and hence more glutamine synthetase. Table 5 shows the results of these experiments. Azaserine did not appear to inhibit growth at concentrations as high as 50 mμg. DON/ml., being more toxic to the organism, began to inhibit growth at concentrations above 5 mμg./ml.; below this concentration growth appeared to be somewhat stimulated by DON. Indeed, as expected, within the concentrations of these compounds which did not inhibit growth, the synthesis of glutamine synthetase was increased. Under the most favourable conditions, the increase amounted to 30% above the control value, a significant though slight increase. Addition of azaserine or DON to the reaction mixture to a final concentration of 13 μg./ml. did not inhibit glutamine synthetase activity.

DISCUSSION

Repression of glutamine synthetase by L-glutamine has been observed in mammalian cells in culture (DeMars, 1958; Paul & Fottrell, 1963), in germinating wheat grains (Rijven, 1961), and in bacteria (Ravel, Humphreys & Shive, 1965). In addition to

L-glutamine, we have found that other amino acids also are capable of decreasing the synthesis of this enzyme. The ability of D-glutamine to repress the enzyme formation in *Escherichia coli* seems to result from its appreciable biological activity as a substrate of the enzyme in the γ-glutamyl transfer reaction. Levintow & Meister (1953) reported that D-glutamine exhibited only about 1 % of the activity of L-glutamine for the enzyme from peas. Hence, D-glutamine, as a substrate in the transfer reaction, is more active for the bacterial enzyme than for the pea enzyme. In contrast, D-glutamate, as a substrate in the synthesis reaction, showed much less activity for the *Escherichia coli* enzyme (Woolfolk *et al.* 1966) than for the pea enzyme (Levintow & Meister, 1953). Apparently, the bacterial enzyme shows low specificity for the L-isomer in the transfer reaction, but high specificity for the L-isomer in the synthesis reaction. The relative stereospecificity displayed by the two reactions of the same enzyme deserves further consideration.

Both ammonia and glutamate are substrates of glutamine synthetase. Hence, induction of the enzyme by glutamate can be readily understood. But contrary to expectation, ammonia represses the enzyme synthesis. Perhaps, in *Escherichia coli*, the synthesis of glutamine serves to a large extent as a storage for nitrogen. When the nitrogen supply is limited, the bacteria respond by converting as much inorganic nitrogen into glutamine as possible. Consequently, the bacteria synthesize more glutamine synthetase. However, when the nitrogen supply is plentiful, there is little need for storage, and the enzyme synthesis is kept at a minimum. Furthermore, we observed an inverse relationship between bacterial growth and the synthesis of glutamine synthetase when ammonia was the sole source of nitrogen and when amino acids other than glutamic acid supplemented ammonium sulphate as the major source of nitrogen. This inverse relationship suggests that the state of nitrogen metabolism of the growing bacteria can affect the synthesis of glutamine synthetase.

The demonstration that azaserine and DON increased the synthesis of glutamine synthetase leads us to speculate that both compounds exert their effect by virtue of their competitive inhibition with glutamine. These antibiotics are competitive inhibitors of glutamine in the synthesis of formylglycinamidine ribonucleotide (Levenberg *et al.* 1957). Thus, high concentrations of glutamine can abolish the inhibition. Possibly the bacteria respond to the presence of the inhibitors by synthesizing more glutamine, and hence more glutamine synthetase. In so doing, the bacteria appear to have succeeded in combating the inhibition, and thereby sustaining growth. Maxwell & Nickel (1957) reported that glutamine, among other amino acids, could relieve the inhibition by azaserine and DON of the growth of *Escherichia coli*.

On the basis of indirect evidence obtained in the study of a partially purified preparation of glutamine synthetase from rat liver with ligands and cations, Wu (1964*b*) deduced that the enzyme might contain a metal ion that inhibited its activity, and that the increase in activity following the addition of certain organic ligands might result from removal of the inhibitory ion originally bound to the enzyme. The recent demonstration by Woolfolk *et al.* (1966) that the crystalline bacterial glutamine synthetase contains Mn^{2+} seems to have substantiated this deduction. Since the results from this and previous (Wu, 1964*b*) studies have shown that, under the conditions which are optimal for glutamine synthesis, Mn^{2+} cannot replace Mg^{2+}, the rat liver enzyme and the *Escherichia coli* enzyme may be alike with respect to their metal-binding.

This work was supported in part by research grants, AM-07319-04 and FR-05383-05, from the National Institutes of Health, U.S. Public Health Service.

This paper is no. VII in the series—Glutamine synthetase.

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J. gen. Microbiol. (1968), **51**, 67–74
Printed in Great Britain

Production of Extracellular Enzymes by *Phytophthora palmivora* (Butl.) Butl.

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(*Accepted for publication* 7 *September* 1967)

SUMMARY

Phytophthora palmivora is more exacting in its nutrition for extracellular enzyme production than for growth. All of thirteen defined media tested supported fairly good growth. The major enzymes detected were: endopolygalacturonase (endo-PG), maceration factor (MF), α-L-arabinofuranosidase (AF) and phenolase. These enzymes were not detected in four of the five natural media tested. In egg-plant extract, traces of endo-PG, MF and pectin-methyl esterase were detected and higher activities of AF and phenolase produced. Inability to detect the enzymes in cultures in other plant extracts was possibly due to their inactivation by the oxidized phenols formed. The inability to detect other enzymes in cultures in defined media, especially those enzymes already implicated in plant diseases, may also be due to the fact that the media tested in the present work were not suitable for their induction. The most suitable basal medium for growth as well as good production of the major enzymes consisted of (%): 1, pectin; 2·5, glucose; 1, asparagine; 0·025, $MgSO_4.7H_2O$; 0·5, Difco yeast-extract; traces of thiamine.

INTRODUCTION

The fungus *Phytophthora palmivora* (Butl.) Butl. is one of the most common pathogens of a considerable number of tropical and subtropical plants in more than fifty genera (Hickman, 1958). It causes wilt of the seedlings, different types of rot of the fruits and other effects. It is best known as a pathogen of cacao (*Theobroma cacao* L.) in which it causes pod rot, commonly called blackpod disease (Thorold, 1967). The first symptom of infection of cocoa pods is a small brown spot with an irregular somewhat water-soaked fringe, but quite firm in texture. The spot gradually increases in size within 3–4 days until the pod turns black and finally dries out. Since the symptom expression is quite different from that of a softer rot, such as that caused by *Sclerotinia fructigena* and *Botrytis cinerea* (Cole, 1956), elucidation of the role of the fungal enzymes in the physiology of the disease might be a useful contribution to the study of the parasitism of *P. palmivora*.

The processes of infection by *Phytophthora palmivora* have been shown on cacao (Orellana, 1953; Spence, 1961), on *Piper nigrum* L. (Holliday & Mowat, 1963), and its pathogenicity recorded on tomatoes (Weststeijn, 1964), on rubber (Peries, 1964) and on a wide range of hosts (Turner, 1960). The physiology of the fungus has also been extensively studied (Weststeijn, 1964; Peries, 1964; Cameron & Milbrath, 1965; Roncadori, 1965). However, little is known about the physiological aspects of the disease and the role played by extracellular enzymes of the fungus. There is now evidence that cell-wall degrading enzymes play an important role when lesions are formed

in plants by bacteria and fungi (Wood, 1959, 1960; Brown, 1955, 1965; Bateman & Millar, 1966) and it seems, therefore, reasonable to assume that such enzymes may be active in the formation of rot incited by *P. palmivora*. Spence (1961) suggested that pectic enzymes might be important in the host/parasite relations, although none of these enzymes were detected in the infected tissue and in the culture media tested. The present study was undertaken to examine the extracellular enzyme production by *P. palmivora* in a range of culture media.

METHODS

Chemicals. Brown Ribbon pectin (an apple pectin) was obtained from Union Crystalex Gelatin, England, and sodium polypectate (sodium ammonium pectate containing 4·2% sodium), and polygalacturonic acid from the Sunkist Growers Inc., California. Other chemicals used were sodium carboxylmethylcellulose (Cellofas B; I.C.I. Ltd.), *o*-nitrophenyl-β-D-galactopyranoside (L. Light, Ltd.), *p*-nitrophenyl-α-L-arabinofuranoside (supplied by A. H. Fielding, Long Ashton Research Station), charcoal gelatin disks (Oxoid), xylan (Nutritional Biochemicals Corporation, Cleveland, Ohio) and peanut meal (prepared as described by Tuttobello & Mill, 1961).

Organisms. The isolates of *Phytophthora* numbered 63552, 63554, 79226, 79239 and 80298 were supplied by the Commonwealth Mycological Institute, Kew; isolates S–O and NP 15 were from the culture collection of the Cocoa Research Institute of Nigeria, and the isolate 'COMUM' was supplied by G. Medeiros of the Cocoa Research Station, Bahia, Brazil. The isolates were all 'cocoa' types; all except 'COMUM' were West African isolates.

Cultural methods. The cultures were maintained on oatmeal agar and stored at room temperature (21°). The organism was sub-cultured on potato glucose agar (PGA) during the work reported here. To obtain inocula for growth experiments subcultures on the PGA plates incubated at 25° for 7–10 days were used. Standard inoculum disks (one for every 5 ml. medium) were then cut with a no. 1 cork-borer from the advancing edge of the colony. Static cultures gave a more consistent result than shaken liquid cultures and were therefore preferred to the latter. The cultures were incubated for 7–10 days at 25°.

Culture media. These were tested for suitability to give good growth as well as for adequate enzyme production. Those previously tested by Spence (1961) were not used.

Defined media. The following were tested:

(i) Per 100 ml.: glucose, 1·0 g.; KH_2PO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·05 g.; $Ca(NO_3)_2$, 1·0 g.; $Fe(NH_4SO_4)_2.6H_2O$, 0·02 mg.; $ZnSO_4.6H_2O$, 0·02 mg.; $MnSO_4.4H_2O$, 0·01 mg. (Roncadori, (1965), for the growth of *Phytophthora* species).

(ii) Per 100 ml.: sucrose, 1·5 g.; pectin, 1·5 g.; KH_2PO_4, 0·1 g. $MgSO_4.7H_2O$, 0·05 g.; $FeSO_4.7H_2O$, 0·001 g. KCl, 0·05 g.; $NaNO_3$, 0·3 g. (modified Czapek medium).

(iii) Per 100 ml.: glucose, 0·2 g.; pectin, 2·5 g.; NH_4NO_3, 2·0 g.; $MgSO_4.7H_2O$, 0·02 g.; KH_2PO_4, 1·0 g. (Pandey & Gupta, (1966) for production of pectic enzymes by *Alternaria tenuis*).

(iv) Per 100 ml.: Glucose, 0·5 g.; pectin, 1·0 g.; asparagine, 0·4 g.; $MgSO_4.7H_2O$, 0·05 g.; KH_2PO_4, 0·1 g. (Pandey & Gupta, 1966).

(v) As in (iv) with 0·3 g. $NaNO_3$ replacing asparagine.

(vi) As in (iv) with 1·5 g. glucose instead of 0·5 g.

(vii) As in (vi) with 0·3 g. $NaNO_3$ for asparagine.

(viii) Per 100 ml.: pectin, 1·6 g.; NH_4 tartrate, 1·5 g.; KH_2PO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·05 g. (Cole (1956) for *Sclerotinia fructigena*).

(ix) Per 100 ml.: peanut meal extract, 2·0 g.; KNO_3, 1·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g. Difco yeast-extract, 0·1 g. (Fuchs, Jobsen & Wouts (1965) for production of arabanases by pathogenic fungi).

(x) As in (ix) with 2·0 g. glucose replacing peanut meal extract.

(xi) Per 100 ml.: pectin, 1·0 g.; asparagine, 0·1 g.; KH_2PO_4, 0·05 g.; $MgSO_4.7H_2O$, 0·025 g.; Difco yeast-extract, 0·5 g.; thiamine, 0·0001 g. (Clarke (1966) for production of enzyme by *Phytophthora infestans*).

(xii) As in (xi) with 2·5 g. glucose replacing pectin.

(xiii) As in (xi) with 2·5 g. glucose supplementing pectin.

Natural media. The following plant extracts were tested: tomatc, potato, cocoa pod, eggplant; 200 g. of each tissue were macerated in 1 l. water for 5 min. in a Waring Blendor, and then strained through muslin. The cocoa-pulp extract was prepared by washing the beans from ripe pods in 1 l. water and then straining through muslin. The potato extract was used with or without glucose supplement. Portions of 25 ml. of each medium were dispensed in 6 oz. medicine bottles, and autoclaved at 121° for 15 min. after the pH had been recorded by glass electrode pH meter (Electronic Instruments Ltd., Richmond, Surrey).

Yield of organisms. Mycelium was harvested on a weighed Whatman no. 41 filter paper, washed with a small quantity of warm distilled water, heated overnight in an oven at about 90°, cooled in a desiccator and weighed. No further loss in weight was obtained by longer periods of drying.

Enzyme preparations. Crude culture filtrate obtained after harvesting mycelium was used as such for enzyme assays or was dialysed overnight against distilled water at 4°. The enzyme solution was stored at 4° until used.

Enzyme assays

Boiled culture filtrate was used as a control in all assays.

Endo-polygalacturonase (*endo-PG*) was examined by the agar cup-plate diffusion technique as described by Dingle, Reid & Solomons (1953). Pectinol-10 M standard solution (0·01 %) was made the reference unit of 100.

Exo-polygalacturonase (*exo-PG*) *activity* was assessed by a colorimetric method described by Hancock & Millar (1965), by measuring the rate of increase of galacturonic acid concentrations in enzyme substrate reaction mixtures, with dinitrosalicylic acid reagent at 575 mμ extinction.

Pectin methyl esterase (*PME*) *activity* was determined by the titration method described by Smith (1958). The enzyme reaction resulted in increased acidity of the reaction mixture which was titrated back to the original pH value by standard alkali.

Polygalacturonate trans-eliminase (*PTE*) *activity* was determined by spectrophotometry as described by Hancock & Millar (1965). The reaction mixtures contained 1 ml. culture filtrate, 4 ml. polygalacturonic acid or sodium polypectate solution (0·25%) in 0·1 M-tris-HCl buffer (pH 8·5), 1 ml. $CaCl_2$ solution at final concentration 10^{-3} M. The liberation of unsaturated bonds during depolymerization of the pectate or polygalacturonic acid was followed at 230 mμ with a spectrophotometer.

The maceration (MF) activity of the culture filtrate was assessed by a method similar to that of Brown (1915) and used by Cole (1956) and Byrde (1957). Cylinders of fully turgid potato-tuber tissue were cut with a no. 6 cork-borer, and disks 0·35 mm. thick cut from these after injection with water under an air pump. Activity was estimated by placing four discs in the test solution and testing at intervals to determine the times at which coherence was lost when the discs were subjected to a gentle pull with two dissecting needles. The maceration activity of the preparation was defined as $100/t$ where t was the time (min.) for the discs to lose coherence.

α-L-arabinofuranosidase (AF) activity was determined by the colorimetric estimation of *p*-nitrophenol liberated from *p*-nitrophenyl-α-L-arabinofuranoside (Fielding & Hough, 1965). The reaction mixture was made up of 0·25 to 1 ml. culture filtrate, 5 ml. acetate (0·1 M) buffer (pH 4·7), 0·5 ml. substrate solution (0·5 mg./ml.). After 1 hr at 25°, 1 ml. saturated Na_2CO_3 solution was added to stop the reaction and to develop the yellow colour. The Spekker readings were taken using a violet filter (Kodak no. 601, wavelength 4300 Å). The activity was expressed in units/ml./hr as absorption readings after applying corrections for absorption of enzyme and substrate blanks respectively.

β-Galactosidase activity was also estimated by a colorimetric method similar to that described for AF, the substrate being 0·1 % *o*-nitrophenyl-β-D-galactopyranoside. A colorimetric method was used to assess polyphenolase activity, a modification of that described by Ponting & Joslyn (1948). The reaction mixture contained 1 ml. culture filtrate, 4 ml. 1 % pyrocatechol in McIlvaine's buffer (pH 7·0), 2 ml. distilled water. The reaction was stopped by adding a few drops of N-HCl. The brown colour intensity of the oxidized substrate was determined at 470 mμ by Spekker absorptiometer.

Cellulase (Cx) was determined by a viscometric method: 1·2 % solution of sodium carboxymethyl cellulose in citric acid NaOH (Sørensen's citrate II) buffer (0·05 M) was used as the substrate.

Other assays for α-amylase, protease, lecithinase and xylanase were by the agar cup-plate method as described for endo-PG activity, with soluble starch, gelatin, lecithin and xylan as substrates, respectively, in 0·2 M-acetate buffer (Dingle *et al.* 1953). The detection of protease activity was also attempted with charcoal gelatin disks as first described by Kohn (1953).

The cup-plate and PME assays were incubated at 25° for 18–24 h.

RESULTS

Enzyme production from growth in natural media

Culture filtrates of *Phytophthora palmivora* isolate 63552 were tested for the activities of thirteen extracellular enzymes, most of which have been implicated in plant diseases. All the natural media were unsuitable for the production of the enzymes except α-L-arabinofuranosidase (*AF*) and phenolase. The addition of glucose to potato extract medium resulted in inhibition of production of all these enzymes. Similar inhibition of enzyme production in other fungi has been reported (Horton & Keen, 1966; Husain & Kelman, 1959).

A similar experiment was made to assess the suitability of these natural media for growth and enzyme production (PG, PME, AF and MF) of five isolates (nos. 63552, 63554, 79239, S–O and NP 15). Within the limits encountered, the pH values of the extracts did not appear to affect the growth of the isolates. Egg-plant fruit extract

supported the best over-all growth (ranging from 50 to 77 mg.) and was also the best medium for AF production by all five isolates (0·53 to 0·67 units/ml./hr.) The isolate with the least growth produced the highest AF, and production of maceration factor MF) was not related to AF production, as suggested by Byrde & Fielding (1965). In other extracts, traces of PME were generally detected, but PG was found only with isolate 63554 on cocoa pulp. Polygalacturonate *trans*-eliminase was one of the many enzymes not detected in these media.

Phenolase was detected in all the media except potato extract + glucose. The action of this enzyme accounted for the browning observed in most of the media and presumably the inhibition of the pectic enzymes.

All the media supported growth to a greater or less extent, potato extract gave the poorest growth of all isolates tested. When glucose was added to potato extract, however, growth was appreciably increased, but the only traces of enzyme detected were with isolates 79239 and S–O. In general, lack of enzyme production did not seem to restrict growth. The above results confirmed that different nutritional factors influenced growth and enzyme production by *Phytophthora palmivora*, and that the medium for best growth was not the most suitable for enzyme production.

Enzyme production in defined media

The culture filtrates of isolate 63552 in defined media were examined for thirteen extracellular enzymes. All the media tested supported adequate growth. The following enzymes were not detected in the culture filtrates: PME, PMG, PGTE, β-galactosidase, α-amylase, protease and lecithinase. The major enzymes detected were endo-PG, AF, MF and phenolase. Endo-PG was detected with all media; AF was detected in media iv, ix, xi and xiii, with traces in the remainder; MF was detected in media (iv) and (ix)–(xiii), with traces in (i), (ii), (iii) and (viii); phenolase was detected in media (ix)–(xiii), and Cx in medium (ix), with traces in (x)–(xiii). Traces of exo-PG were detected in media (ii) and (iii).

Suitability of defined media for production of the major enzymes by Phytophthora palmivora isolate 63554

All the defined media tested supported fairly good growth of isolate 63554 (Table 1). The best medium for growth was again not necessarily the best for enzyme induction; media (xiii), (xi), (ix) and (xii) were the best (in that order) for growth; while the highest PG production was in media (ii), (vi), (vii), (ix) and (xiii).

The activities of the enzymes detected were not related to the growth yield of isolate 63552, nor to the pH value of the media used.

DISCUSSION

From the previous work (Spence, 1961; Roncadori, 1965), it seems likely that *Phytophthora palmivora* is more exacting in its nutrition for enzyme production than for growth. The present study thus indicates that the media tested by Spence (1961) were unsuitable for enzyme production, although these might have supported good growth. Not all the defined media tested in the present work gave production of the enzymes examined, although they all supported fairly good growth (Table 1).

Although many fungal extracellular enzymes have been connected directly or indirectly with pathogenesis of plant diseases, only three major enzymes of the thirteen assayed could be detected; these were detected in culture filtrates of all isolates tested.

Phenolase was also detected in the culture filtrates and may be important in plant infection and symptom expression (Deverall & Wood, 1961; Oku, 1966). The three major enzymes were not detected in cultures grown on the natural media used, except traces of AF activity with isolate 63552 in potato and cocoa-shell extracts, and with isolates 63554 and 79239 in cocoa-shell extract. The absence of enzyme activity, except phenolase, in the natural media culture filtrate was probably due to inhibition by oxidized phenolics (Williams, 1963); most of the natural media turned brown. This may be the main factor for the absence of pectic enzymes in diseased tissues.

Table 1. *Comparative suitability of different synthetic media for growth and enzyme production of Phytophthora palmivora isolate 63554*

(Averages of three determinations)

Media	pH		Dry wt. (mg.)	Enzyme production*		
	Initial	Final		PG	AF	MF
(i)	4·30	4·10	41	21·9	0·009	0
(ii)	5·50	4·40	19	69·2	0·004	0
(iii)	5·10	4·75	20	21·9	0	0
(iv)	5·35	6·85	15	25·1	0·031	1·5
(v)	5·55	4·50	20	39·8	0	—
(vi)	5·35	6·80	29	39·8	0	—
(vii)	5·55	4·50	18	25·1	0	—
(viii)	5·80	5·50	38	0	0	0
(ix)	5·50	5·50	89	50·1	0·002	1·9
(x)	4·85	4·70	66	14·5	0	1·4
(xi)	4·75	4·55	106	31·6	0·104	1·3
(xii)	5·95	4·80	82	15·9	0	1·6
(xiii)	4·80	4·90	130	39·8	0·184	1·2

* For units see Methods section.

Products of PG activity in some host-parasite relations activate the phenolase which in turn oxidize the host phenolic compounds; the latter inhibit the PG activity and the overall process results in lesion formation (Deverall & Wood, 1961; Oku, 1966). Phenolase has been detected both in the culture filtrate of *Phytophthora palmivora* and in diseased tissues; a much lower activity was detectable in healthy plant extracts.

The detection of traces of pectin methyl-esterase in culture filtrates from natural media, particularly in eggplant extract, may be significant in the pathogenicity of the species. Clarke (1966) has suggested that this enzyme may be important in the host-parasite relationship of *Phytophthora infestans*, and Smith (1958) found indications that its production by various *Xanthomonas* species was correlated with their pathogenicity.

In conclusion, the best basal medium for the production of the major enzymes detected consisted of 1% pectin, 2·5% glucose, 1% asparagine, 0·05% KH_2PO_4, 0·025% $MgSO_4.7H_2O$, 0·5% Difco yeast extract and traces of thiamine.

The work in this paper was done under the kind supervision of Dr R. J. W. Byrde, and the author is grateful for his interest and the helpful criticism during the preparation of this manuscript. Thanks are also due to Professor H. G. H. Kearns for

making available the research facilities on the Station; to Mr A. H. Fielding and Miss Celia Slowley for assistance, and the Cocoa Research Institute of Nigeria for financial support.

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J. gen. Microbiol. (1968), **51**, 75–84
With 1 plate
Printed in Great Britain

Streptomycin Resistance and Morphological Variation in *Phytophthora cactorum*

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(*Accepted for publication* 11 *September* 1967)

SUMMARY

Strains of *Phytophthora cactorum* resistant to streptomycin were obtained by incubating zoospores in liquid medium containing the drug. One of the strains required streptomycin for growth. In this dependent strain and one of the resistant strains, the dependence or resistance was transmitted to both asexual and selfed sexual progeny without any segregation for sensitivity. Change from dependence to resistance occurred with low frequency. Some of the resistant and the dependent strains showed much greater morphological variation than the wild type. Continuing segregation for morphological characters was observed during vegetative growth and in the asexual progeny of single zoospores, and the ability to segregate was not lost following sexual reproduction (selfing). Cultures were more variable after storage at 3°. There was evidence suggesting a particulate nature for the determinants of these morphological differences, normal zoospores having one such determinant.

INTRODUCTION

Virtually nothing is known of the genetics of any of the oomycetes, among them the genus *Phytophthora*, which includes many important plant pathogens. The most obvious reason for the neglect of these fungi by geneticists is the difficulty in germinating oospores and so of obtaining sexual progeny. Hence not only do we not know the basis of the interesting examples of variation in morphology and pathogenicity and of the complex mating behaviour which have been described in various phytophthoras; we do not even know for certain at what point in the life history meiosis occurs, and whether the somatic hyphae are haploid or diploid. Recent cytological evidence from species of the genera *Pythium*, *Phytophthora* and *Achlya* (Sansome, 1961, 1963, 1965) would seem to indicate that meiotic divisions take place in the gametangia just before fertilization. If this were so the life-cycle in these species and possibly in all oomycetes would be based on the diplontic pattern. As interpretation of division figures in these fungi is extremely difficult, genetical proof of this hypothesis is necessary. Mullins & Raper (1965) obtained data on the inheritance of maleness and femaleness in Achlya and Dictyuchus, which they interpreted as indicating that the somatic hyphae are diploid. However, these characters are complex, and a study by using simple gene markers is desirable.

Attempts to obtain 'biochemical' mutants of Phytophthora (e.g. Buddenhagen, 1958; Clarke & Robertson, 1966) have not been successful. This might be due to technical matters; but alternatively, if these fungi are diploid, as Sansome suggested,

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the lack of success would be understandable, as such mutants in Aspergillus and yeast are always recessive, and so would not be expected to reveal themselves until after recombination. On the other hand, mutations to drug resistance have often been found to be dominant or semidominant (Roper & Käfer, 1957; Warr & Roper, 1965; Middlekauf *et al.* 1957) and so could be selected directly in a diploid. We therefore set out to select drug-resistant mutants of *Phytophthora cactorum* (Leb. & Cohn) Schroet., and this paper reports a study of streptomycin resistance.

Unfortunately we have as yet no genetical information about the resistant strains obtained. We have succeeded in germinating oospores in large numbers (Shaw, 1967), but the fungus is homothallic, and in making a cross one would need to be able to distinguish hybrid sexual progeny from selfed progeny by their showing recombination between markers from the two strains; such markers are not yet available. However, if the strain were diploid and heterozygous for a dominant gene conferring resistance, its selfed progeny would segregate; if the strain were haploid, no segregation would be expected. If the resistance were due to a cytoplasmic condition, either no segregation or non-Mendelian segregation might occur. It is therefore of interest to compare the sexual progeny of the resistant strains, resulting from the germination of oospores, with their asexual progeny, resulting from zoospores.

METHODS

The strain of *Phytophthora cactorum* used was IMI 21168, obtained from the Commonwealth Mycological Institute, Kew. Incubation was at 24° in the dark.

Standard medium was made up from two separate solutions, which were kept unautoclaved in polythene bottles at −15°. The first solution contained (g.): sucrose, 10; L-asparagine, 1·0; $MgSO_4.7H_2O$, 0·25; KH_2PO_4, 0·5; thiamin hydrochloride, 1 mg.; trace element solution, 1 ml.; water, 250 ml. (The trace element solution contained $Na_2B_4O_7.10H_2O$, 88 mg.; $CuSO_4.5H_2O$, 393 mg.; $Fe_2(SO_4)_3.9H_2O$, 910 mg.; $MnCl_2.4H_2O$, 72 mg.; $Na_2MoO_4.2H_2O$, 50 mg.; $ZnSO_4.7H_2O$, 4403 mg.; EDTA, 5 g.; water, 1 l.). The second solution was made by boiling 75 g. frozen garden peas in 250 ml. water and filtering off the solid matter. For use the two solutions were thawed, and 500 ml. water added and autoclaved at 120° for 15 min.

Standard medium agar (SMA): 8·0 g. Oxoid Agar no. 3 was added to 1 l. standard medium.

Medium for producing oospores was SMA to which was added 1 % (w/v) of an oat extract made by refluxing ground oats in light petroleum for 1 hr, filtering, and evaporating the solvent from the filtrate.

Pea meal agar. Frozen garden peas, blended, 300 g.; agar, 8 g.; water, 1 l.

Soft non-nutrient agar. Agar, 8 g.; water, 1 l.

Sucrose salt solution for suspending zoospores (g.): sucrose, 20; NaCl, 0·215; KCl, 0·0075; $Na_2S_2O_3.5H_2O$, 0·05; CaCl, 0·012; in 1 l. water.

Streptomycin (as Streptomycin Sulphate, Glaxo) was dissolved in sterile water and added to media after autoclaving.

Production of zoospores. Zoosporangial cultures were prepared from 8-day colonies grown on pea meal agar. Three strips of mycelial mat (7 × 0·5 cm.) were cut from the surface of a colony and transferred with a minimum of adhering agar to a 9 cm. Petri dish of soft non-nutrient agar. Zoosporangia were formed in these strip cultures within 72 hr. They were induced to discharge zoospores by flooding with 5 ml. de-ionized

water at 15° and incubation at 15°. To prevent the zoospores bursting soon after discharge, 2·5 ml. 4 % (w/v) sucrose at 15° was added 30 min. after the initial flooding with water and a further 2·5 ml. at 15 min. later, thus bringing the final sucrose concentration to 2 %. The cultures were then removed to room temperature (20–22°) and the zoospores harvested.

High viability (above 50 %) of zoospores in suspensions of low concentration could only be maintained by adding physiological salts to suspensions in sucrose. Zoospore suspensions were diluted into the sucrose salt solution (see above), and viability was then assured for periods of 2–3 hr.

This method of producing zoospores was modified when zoospore progeny of a large number of isolates was required. The fungus was grown on discs of pea meal agar 4 mm. diam. and 8 mm. thick in a moist chamber for 72 hr, after which each disc was transferred to a 1 oz. bottle or culture tube containing about 10 ml. of soft non-nutrient agar. After a further 72 hr of incubation, zoospore suspensions were obtained by flooding with 0·5 ml. water at 15° and subsequently adding two lots of 4 % sucrose each of 0·25 ml. as before. By this method the amount of pre-sporulation growth was considerably reduced.

Germination of oospores was as described by Shaw (1967).

Measurement of growth. In many cases colonies on agar medium were too variable in density for their diameters to be a good measure of differences between them in growth. However, the light absorption of a whole colony was considered to be a suitable measure of growth. Ten colonies were grown in a 9 cm. Petri dish, to a size not exceeding 15 mm. diam. A Zeiss photomicrographic apparatus which included a photocell connected to a meter, was set up on a microscope stand with a high-intensity lamp. The condenser, objective and eyepiece of the microscope were removed. The light intensity was adjusted to give full-scale deflection on the meter on an uncolonized part of the dish, and the light absorbed by each colony in turn read off.

RESULTS

Effect of streptomycin on wild type

Figure 1 shows the effect of streptomycin on growth of wild type. From each of 50 colonies resulting from a plating of wild type zoospores on SMA, five inocula about 1 mm. cube were cut and transferred to SMA + streptomycin 0, 3, 7·5, 20 and 100 μg./ml. Growth was measured photometrically and the histograms show the amounts of growth, grouped in arbitrary but equal intervals according to light absorption. Growth rate, measured by increase in colony diameter, was decreased to half the control value with streptomycin 7 μg./ml.

Zoospores plated directly on SMA with streptomycin 20 or more μg./ml. produced germ tubes up to 1·5 mm. long with only a few lateral branches (Fig. 2); further growth was inhibited.

Selection of resistant strains

Each of four 2 l. flasks containing 500 ml. liquid standard medium + streptomycin 100 μg./ml. was inoculated with 10^5 zoospores and incubated without shaking or stirring for 10 days. Eight growths appeared (not all in the one flask) and were transferred to SMA + streptomycin 100 μg./ml. To test the stability of streptomycin tolerance in the absence of drug and in zoospore progenies, each isolate was treated as follows:

(i) Plates of pea-meal agar were inoculated to provide mycelium for strip cultures. (ii) Zoospores released from strip cultures were plated on SMA and on SMA + streptomycin 100 μg./ml. (iii) Six spores germinated on SMA and six on SMA + streptomycin were transferred to SMA without streptomycin. (iv) Inocula from each of these twelve cultures were tested on SMA + streptomycin 0, 100, 250 and 500 μg./ml.

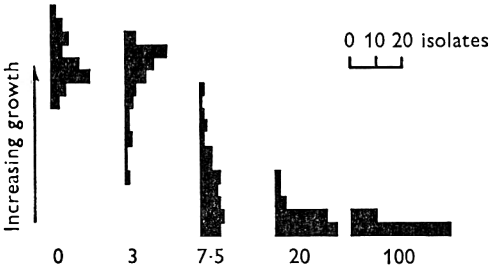

Fig. 1

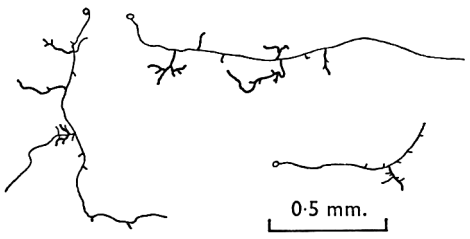

Fig. 2

Fig. 1. Effect of streptomycin (concentrations 0, 3, 7·5, 20, 100 μg./ml.) on growth of wild-type *Phytophthora cactorum*. Histograms show variation in response of 50 single zoospore isolates to each concentration. Vertical axis represents light absorption by colonies after growth from similar inocula for equal times, in grouped arbitrary units.

Fig. 2. Extent of growth in 2 days from wild-type zoospores of *Phytophthora cactorum* on medium containing streptomycin 20 μg./ml.

Two isolates did not sporulate well and were discarded at stage (ii).

The results with the remaining six isolates were as follows:

(*a*) With three isolates, all twelve zoospore cultures grew well on all concentrations of streptomycin and on drug-free medium. These strains were uniformly resistant.

(*b*) With one isolate, the twelve zoospore cultures showed differences in tolerance and this occurred independently of whether they had been germinated on medium with or without streptomycin.

(*c*) With one isolate, all twelve zoospore cultures were uniformly sensitive, like the wild type. Apparently tolerance to streptomycin was only temporary and was lost during growth on drug-free medium.

(*d*) One isolate was streptomycin-dependent, and did not grow on the pea meal agar [stage (i)] unless this was supplemented with streptomycin. The 12 single zoospore cultures of this isolate, six of which were rescued from streptomycin-free medium before growth ceased, were all uniformly dependent.

One of the resistant isolates and the dependent isolate were studied in detail; they were designated *Sr* and *Sd*, respectively.

Inheritance of resistance and dependence

In asexual progeny. The growth of 50 single zoospore cultures of *Sr* and *Sd* on various concentrations of streptomycin was examined. Zoospores of *Sd* had to be grown on medium containing streptomycin, since on SMA growth ceased soon after production of a short branched germ tube (and it was not resumed when suitable amounts of streptomycin were added). To prevent streptomycin being carried over to drug-free medium in the inocula of *Sd*, zoospores were grown into small colonies on SMA + streptomycin 100 μg./ml. and disc inocula from these were transferred to SMA where sufficient growth took place to provide drug-free inocula for testing on various

drug concentrations. Zoospores of *Sr* were germinated directly on SMA. Inocula from 50 single-zoospore isolates of each strain were grown on SMA + streptomycin 0, 10, 100 and 1000 μg./ml. and the growth measured photometrically. Results are shown in Figs. 3 and 4.

In sexual progeny. The results obtained with the above 50 zoospores were designed to serve as a standard of variation to test whether there was any segregation for drug resistance in the sexual progenies of these strains. Oospores resulting from selfing were germinated on plain agar and transferred to SMA and SMA + streptomycin

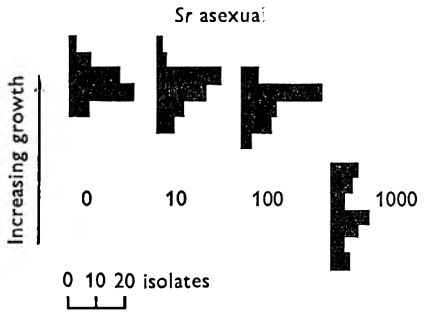

Fig. 3

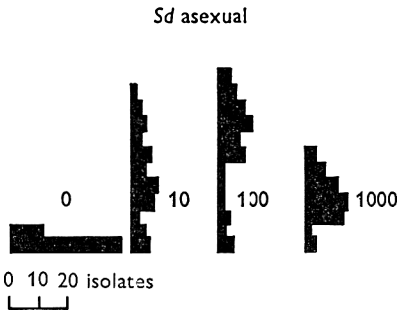

Fig. 4

Figs. 3, 4. Comparison of growth responses of asexual progenies of *Phytophthora cactorum* strains *Sr* and *Sd* to streptomycin 0, 10, 100 and 1000 μg./ml. Fig. 3. Variation in growth of 50 single zoospore isolates of *Sr*. Fig. 4. Fifty single zoospore isolates of *Sd*. Vertical axis represents light absorption by colonies grown in any one experiment from similar inocula for equal times; light absorption values in grouped arbitrary units.

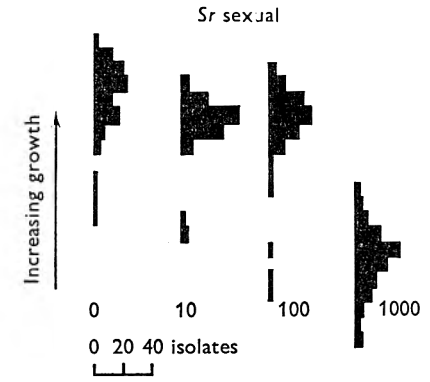

Fig. 5

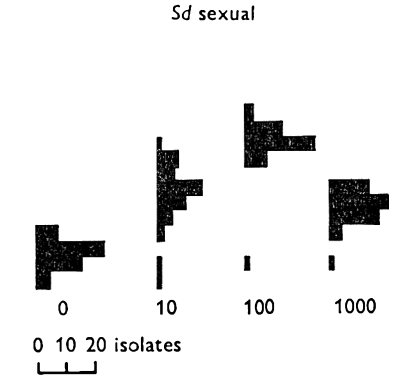

Fig. 6

Figs. 5, 6. Comparison of growth responses of selfed sexual progenies of *Phytophthora cactorum* strains *Sr* and *Sd* to streptomycin 0, 10, 100 and 1000 μg./ml. Fig. 5. One hundred single oospore isolates of *Sr*. Fig. 6. Fifty single oospore isolates of *Sd*. Vertical axis as in Figs. 3 and 4.

100 μg./ml. Colonies from *Sr* oospores developed on both media, but growth from *Sd* oospores occurred only on SMA + streptomycin. Drug-free inccula were prepared from 50 germinated *Sd* oospores and 100 *Sr* oospores in the same way as for zoospores and tested on the same four concentrations of streptomycin. The results of measurements of growth, made photometrically, are shown in Figs. 5 and 6.

The curves showing the variation in growth at each concentration are essentially unimodal; there is no evidence of more than one kind of response to streptomycin in either of these progenies. The response is essentially the same as that of the zoospore progenies. (In a few cases the inoculum did not 'take' quickly but these developed later. This also occurred with wild type.) Thus no segregation occurred during sexual reproduction in either strain.

Reversion of streptomycin-dependence to resistance

When plates of SMA were inoculated centrally with a disc cut from a colony of *Sd* growing on SMA + streptomycin 100 μg./ml., initial growth compared favourably with that made by similar discs placed on medium with streptomycin 100 μg./ml., but presumably with the exhaustion of the streptomycin in the inoculum, growth slowed down and eventually stopped. With an inoculum disc 4 mm. diam. the colonies attained a diameter of approximately 40 mm. However, some 20 days after the initial inoculation, sectors were observed to arise from the edge of such colonies. These non-dependent sectors appeared in one case in all of 6 plates.

It was further found that when zoospores from *Sd* cultures were added to flasks of liquid standard medium without streptomycin, non-dependent colonies appeared with a frequency of about 3×10^{-5} per zoospore.

These reverted strains, which could grow in the absence of streptomycin, could still tolerate streptomycin at 1000 μg./ml. The change was from dependence to resistance.

Morphological change in the resistance strains

Spontaneous change in colony morphology is well known in Phytophthora (Leonian, 1925; Stamps, 1953). In the wild-type strain used here, sectors of different morphology were observed in occasional colonies, and in some platings of zoospores a few variants were noted, but this parent strain is comparatively stable. In striking contrast were the variety of morphological types and the frequency of their occurrence in some of the streptomycin-resistant isolates.

The morphological variation in the streptomycin-dependent strain, *Sd*, growing on SMA + streptomycin 100 μg./ml., was particularly distinct, and was studied in detail. Three morphological types occurred in the first plating of zoospores and were distinguished as follows (Pl. 1, fig. 1 *a*, 1 *b*): (i) *plumulose*, *p*, with the same morphology as wild type; (ii) *knotted*, *k*, which differed from *p* in its rate of increase in colony diameter (on SMA + streptomycin it grew twice as fast as *p*), in its more diffuse growth (with long leading hyphae and short knotted laterals) and in its lack of aerial mycelium: growth as measured photometrically, however, did not differ from *p*; (iii) *regular*, *r*, with growth rate similar to *p*, but with a more even edge to the colony and with much more dense aerial mycelium.

On plating zoospores from each of these types, *k* and *r* were found to be stable, but *p* gave 33–40 % *k* segregants. Plumulose (*p*) colonies sometimes showed *k* sectors. Later it was found that *k* colonies which had risen from *p* gave a small proportion of *p* in their asexual progeny.

The nature of this variation between *p* and *k* was investigated further by comparing the proportions of *p* and *k* type colonies in asexual progeny of different single zoospores. The amount of vegetative growth was kept to a minimum by using the small discs of medium to produce zoospores. One disc was inoculated with mycelium from a

single zoospore colony of *p* type, and from this culture zoospores were obtained and plated on SMA + streptomycin. After incubation for 6 hr, 60 germinated spores were picked off on to discs. In addition, 6 discs were inoculated with a mass of mycelium from the original disc. Zoospores were obtained from most of the disc cultures and plated out, and for comparison, platings were made of zoospores from the six mycelial cultures. The proportion of *k* type was determined in each of these progenies, which numbered from 14 to 272, with an average of about 140.

The results are shown in the histogram (Fig. 7). There were three kinds of progenies from single zoospores: (i) predominantly *p*, but including up to 6% *k* (25 progenies) (Pl. 1, fig. 2); (ii) predominantly *k*, but including up to 5% *p* (22 progenies) (Pl. 1, fig. 3); (iii) intermediate types (2 progenies) with 30 and 33 %*k*, which resembled the progeny of the mycelial mass cultures.

Table 1. *Effect of zoospore size on the proportion of k type segregants from Phytophthora cactorum morphological type p*

Zoospore diameter (μ)	No. of colonies	Percentage *k* segregants
> 20	39	13
14–20	40	45
< 14	40	70

$\chi^2_{[2]} = 26{\cdot}5$, *P* very small.

Table 2. *Effect of storage at 3° on the incidence of k type morphologies in the asexual progeny of Phytophthora cactorum p cultures*

Days at 3°	0	4	8	16	32
Percentage *k* phenotypes*	50·6	55·4	59·2	69·9	61·7

Analysis of variance†

	D.F.	Mean square
Between times		
Linear regression	1	122·35‡
Remainder	3	62·32
Replicates	10	18·78

* Means of three replicates. Percentages based on counts of from 107 to 232 colonies.
† Percentages transformed to angles.
‡ Significant at 5% level.

These results were obtained with the streptomycin-dependent strain growing on SMA + streptomycin 100 μg./ml. When later reverted non-dependent resistant strains were obtained (see above), it was shown that the observed segregation was inherent in the fungus and did not require the continued presence of streptomycin in the medium.

The segregation ratio observed does however depend on the size of the zoospores. When, instead of pea meal agar, SMA with added cholesterol (30 μg./ml.) was used as the pre-sporulation medium, zoospores of various sizes were obtained, the larger ones being multiflagellate and multinucleate. Such a suspension was obtained from a *p* culture and plated on SMA + streptomycin. After 6 hr single germinated zoospores were

isolated and classified in three groups according to size. The proportion of *k* segregants in each class was determined after the zoospores had grown for 72 hr on SMA+ streptomycin; the results are shown in Table 1.

Change from *p* to *k* was also found to occur during storage of cultures under liquid paraffin. The effect of storage in the cold was studied in the following experiment. Pea-meal agar discs were inoculated with a *p* strain and incubated for 72 hr. All except three discs were then transferred to 3°; these three discs were transferred immediately to non-nutrient agar to produce three separate zoospore suspensions, which were plated, and after 72-hr. incubation the colonies were scored for morphological type. Three discs were removed from storage at intervals, zoospores produced, and the percentage of different morphological types in the resulting colonies scored. The results (Table 2) show a significant change in the incidence of *k* types with time of storage.

Oospores from both *p* and *k* cultures were germinated. As in the asexual progenies, a proportion of *k* segregants appeared in the sexual progeny of *p*, and a proportion of *p* segregants appeared in the sexual progeny of *k*. The asexual progeny of two single oospore cultures of *p* phenotype both continued to segregate for *k*. The ability to segregate is not lost during sexual reproduction.

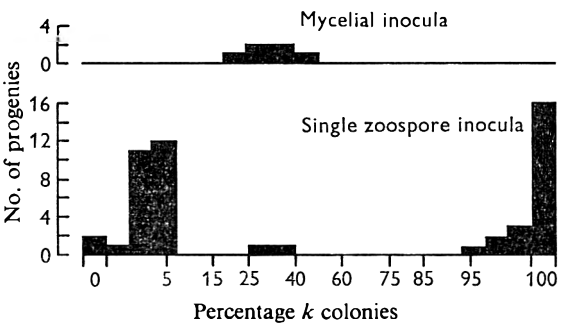

Fig. 7

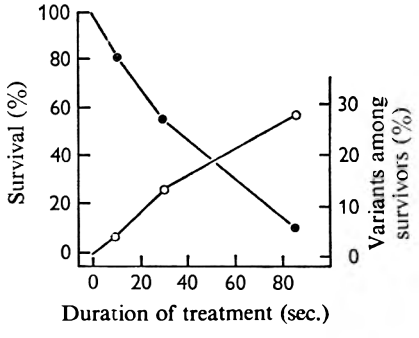

Fig. 8

Fig. 7. Segregation for morphological types *p* and *k* in asexual progeny of *Phytophthora cactorum*. Upper histogram, distribution of frequency of *k* types in progeny of mass mycelial cultures. Lower histograms, distribution of frequency of *k* types in progeny of single zoospore cultures. Progenies with 0 and 100% *k* shown separately, those with other values grouped in arbitrary equal intervals after transformation of percentages to angles.

Fig. 8. Survival of zoospores of *Phytophthora cactorum Sr* after treatment with streptomycin (1000 μg./ml.) [●——●], and incidence of morphological variants among the survivors [○——○].

The induction of morphological change by streptomycin

The question arises as to whether the morphological variants observed in some of the streptomycin-resistant strains were the result of mutagenic action by streptomycin. This was examined by treating zoospores with a high concentration of streptomycin. Since wild-type zoospores are inhibited by streptomycin 20 μg./ml., the resistant strain, *Sr*, was used. This has wild type morphology, is comparatively stable and the spores withstand streptomycin 1000 μg./ml.

A zoospore suspension containing streptomycin 1000 μg./ml. was prepared by adding 1 ml. of the sucrose-salt solution containing 10 mg. streptomycin to 9 ml. of zoospore suspension (about $1{\cdot}5 \times 10^4$ zoospores/ml.); 1 ml. of sucrose-salt solution

was added to 9 ml. of control suspension. Samples were taken from the treated suspension after 10, 30 and 85 min., suitably diluted and then plated. One plating of the control suspension was made. Streptomycin was added to the media in such quantities as to ensure that after plating the zoospores, all Petri dishes contained equal amounts of streptomycin (about 1·5 μg./ml). The number of variant colonies in the survivors was scored after 72 hr, and is shown in Fig. 8. Some of the morphological variants produced by this treatment are shown in Pl. 1, fig. 5, and the control plating in Pl. 1, fig. 4.

On subculture of a number of these variants to SMA, all reverted to wild-type morphology, and the zoospore progeny of 19 variant colonies were also like wild type. Evidently the changes induced by streptomycin in this experiment were not hereditary, or were lost by selection of wild type.

Wild type was grown on SMA + streptomycin 5 μg./ml. and *Sr* on SMA + 500 μg./ml. for 5 weeks (cultures being transferred weekly). Zoospores were then produced and plated on SMA. The numbers of variants observed in these zoospore progenies did not exceed those in control platings from cultures grown on SMA for corresponding periods.

DISCUSSION

Streptomycin resistance and dependence are well known in bacteria. The higher fungi, however, are insensitive to streptomycin; the resistant and dependent strains we have obtained in the streptomycin-sensitive fungus *Phytophthora cactorum* are therefore of interest. The reversion of dependence to resistance is also noteworthy. Unfortunately there is no indication as to the nature of the changes which took place when these strains arose. The resistance and dependence are stable through asexual and sexual reproduction; no segregation was observed in selfed progeny. Resistance might be the result of a change either in a gene (in which case the implication is that the fungus is haploid) or in a cytoplasmic entity. Both genic and cytoplasmic streptomycin resistance are known in Chlamydomonas (Sager, 1954).

It was found that exposure to high concentrations of streptomycin for short periods induced phenotypic change, but in this experiment the change was only temporary. However, it cannot be ruled out that the hereditary morphology changes observed in many of the streptomycin-resistant strains were induced by the drug (cf. Sager & Tsubo, 1962; Sager, 1962).

The heritable variation in morphology we observed has the following characteristics: differences between strains in the degree of variability; continuing segregation during vegetative growth and in the asexual progeny of single zoospore cultures; increased variability with storage; continuance of the ability to segregate following sexual reproduction. Many of these features were also described by Leonian (1925) and Stamps (1953). In the *Sd* strain, zoospores ordinarily have the potentiality to develop into colonies which have one or other of the alternative morphologies, *p* and *k*, and which give nearly uniform progenies. This suggests that *p* and *k* are determined by particulate factors of which each zoospore has one. Large zoospores, which give a high proportion of both morphological types in their progeny, may have two or more of these factors. These factors are apparently mutable, but whether they are nuclear genes or unstable cytoplasmic entities cannot be determined in the absence of known nuclear gene markers.

We wish to acknowledge with thanks the grant of a Science Research Council studentship to D.S.S.

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EXPLANATION OF PLATE

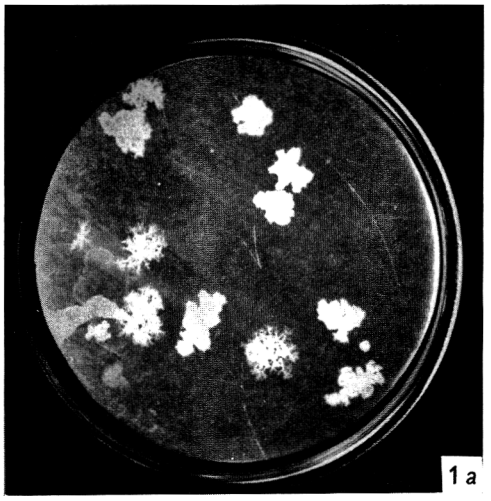
Fig. 1*a*, 1*b*. Plating of *Phytophthora cactorum* strain *Sd* showing the three morphological types, *p*, *k* and *r*.

Fig. 2. Class (i) progeny of a single zoospore of *P. cactorum Sd*; mainly type *p* but showing four colonies of type *k*.

Fig. 3. Class (ii) progeny of a single zoospore of *P. cactorum Sd*; mainly type *k* but showing one colony of type *p* (arrow).

Fig. 4. Plating of untreated zoospores of *P. cactorum* strain *Sr*.

Fig. 5. Plating of zoospores of *P. cactorum Sr* treated with streptomycin (1000 μg./ml.) for 85 min.



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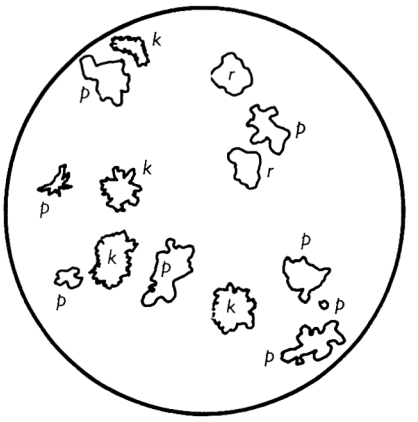

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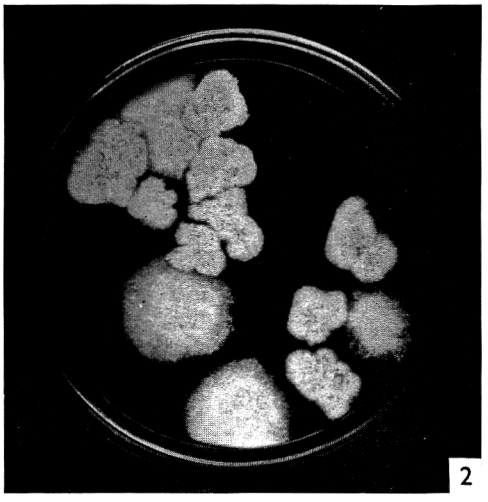

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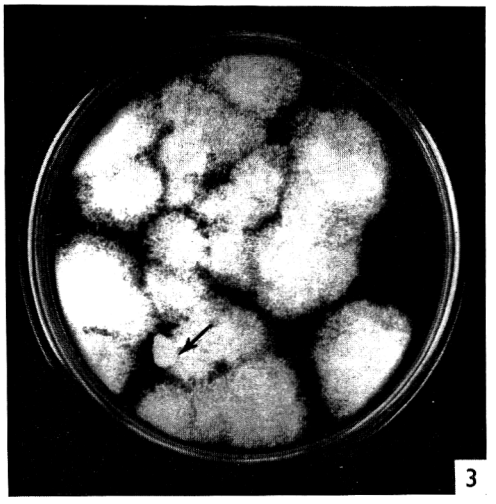

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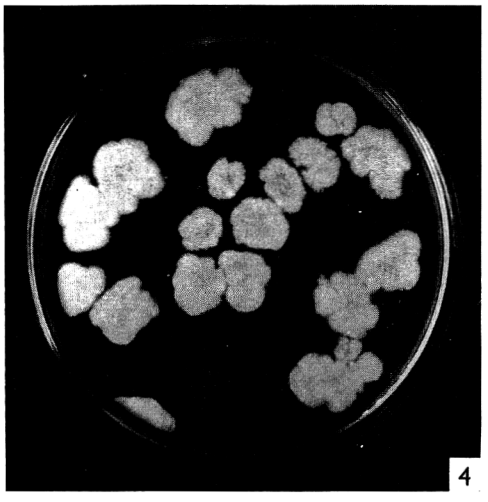

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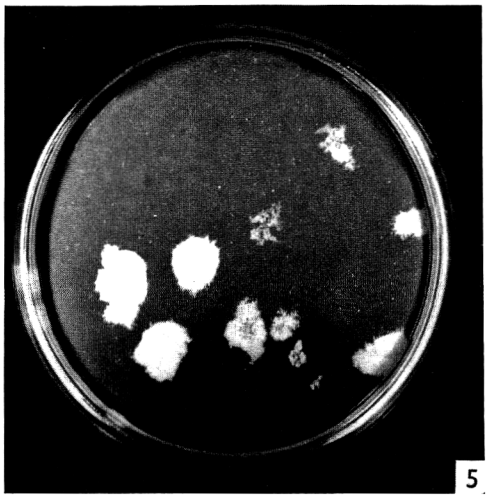

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J. gen. Microbiol. (1968), **51**, 85–95
Printed in Great Britain

Competence in *Bacillus subtilis* Transformation System

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(*Accepted for publication* 12 *September* 1967)

SUMMARY

Changes in competence of *Bacillus subtilis* 168 *try*⁻ in relation to generation time and under other conditions were examined. When the generation time increased or decreased from that associated with maximum competence, competence decreased. In a culture in balanced growth, competence remained at the same level for long periods. In supplemented medium, more bacteria became competent than in minimal medium.

Aeration stimulated the development of competence which reached a higher peak than in an unaerated culture. When the medium was inoculated with spores, competence also developed. The smaller the inoculum of spores, the later the appearance of competence.

Waves of competence were often seen along the main curve of competence, and were influenced by the compositions of the media and by the inoculum size.

During the growth cycle in a closed culture the bacterial cells became competent and then lost this property. Competence was thus a transient state.

The optical density of the bacterial suspension was a good guide to the development of competence, but varied with the strains used and with the cultural conditions.

INTRODUCTION

Many bacterial species are able to take up deoxyribonucleic acid (DNA) when in a state of 'competence' and can then be transformed (Alexander & Redman, 1953; Klein & Klein, 1953; Balassa, 1954; Bracco, Krauss, Roe & MacLeod, 1957; Corey & Starr, 1957; Hotchkiss, 1957; Spizizen, 1958; Goodgal & Herriott, 1961; Catlin & Cunningham, 1964). The DNA derived from bacteriophage (Romig, 1962; Green, 1964; Okubo, Strauss & Stodolsky, 1964; Reilly & Spizizen, 1965) or virus (Abel & Trautner, 1964; Bayreuther & Romig, 1964) can similarly be incorporated in competent bacteria where it can replicate.

Competence is often short-lived and occurs only under special growth conditions. Many factors influence its appearance and therefore its nature has not yet been well defined. One of the important factors is the generation time and experiments have been carried out to investigate in detail changes of competence in relation to generation time. The connexion between competence and synchrony of cell division was also investigated.

METHODS

Bacterial strains. In the transformation experiments the recipient strain was *Bacillus subtilis* 168 *try*⁻, and the donor strain *B. subtilis* MARBURG. Both were kindly provided by Dr B. S. Strauss.

Media. Bacterial strains were maintained on potato agar (Spizizen, 1958; Nester,

1964). *Bacillus subtilis* 168 *try*⁻ was precultivated on minimal glucose yeast agar slope (MGY agar). The development of competence was investigated in MGY liquid medium. T-medium was used for transformation, and MG-agar for the selection of transformants: both media are described by Horváth (1967).

Transforming DNA of *Bacillus subtilis* MARBURG was prepared by the phenol extraction method of Saito & Miura (1963; Horváth, 1967).

Determination of competence and the transformation procedure were previously described in detail (Horváth, 1967).

Cultivation of the bacterial suspensions were carried out in a water bath at 37° on a reciprocal shaker at 100 rev./min. During growth, samples were taken and assayed for the number of competent bacteria by transformation to prototrophy under standard conditions. The number of transformants was calculated from two replicate measurements.

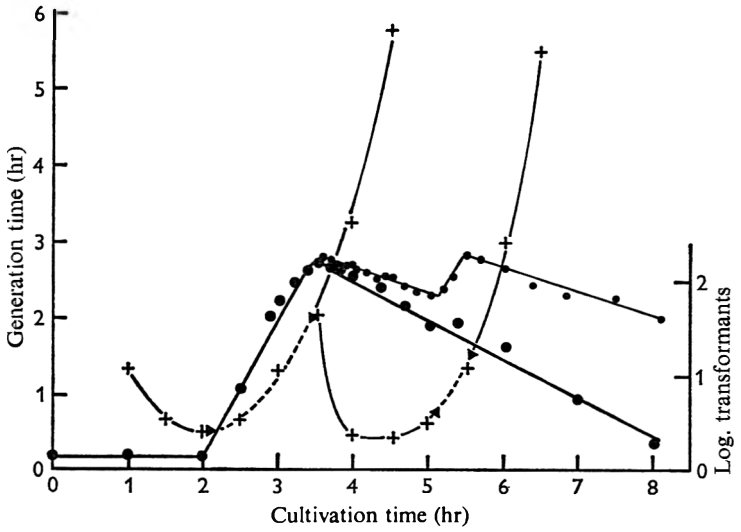

Fig. 1. Correlation between generation time and competence during the cultivation in two flasks. The thin lines represent the values in the second flask. The dotted lines along the curves of the generation time represent the rise phase of competence. ●——●, Competence; +——+ generation time.

Balanced growth was obtained by diluting the bacterial suspension with MGY liquid medium every 10 min. The optical density (OD) then remained 'almost constant' for long periods during cultivation. After diluting the bacterial suspension sufficient culture was discarded to maintain the original volume of the culture.

Chain formation was determined by microscopy and counts analysed statistically by expressing the number of bacteria per chain in $\log_2$. The number of bacterial chains was expressed in per cent (Horváth, 1967).

RESULTS

Correlation between generation time and competence

The preceding paper (Horváth, 1967) showed that the generation time played an important role in the development of competence. In the following experiment *Bacillus subtilis* 168 *try*⁻ was cultivated and the generation time of the bacterial suspension

changed from time to time during incubation. $5{\cdot}9 \times 10^7$ cells/ml. of OD about 0·1 were inoculated into 10 ml. MGY liquid medium in a 100 ml. Erlenmeyer flask fitted with side arm for OD measurements. During subsequent incubation and growth, samples were assayed for the number of competent bacteria by measuring the efficiency of transformation to prototrophy under standard conditions. The optimal competence was determined according to OD value. When the bacterial suspension showed optimal competence a small portion was put into a second Erlenmeyer flask and diluted with MGY liquid medium to 0·1 OD value. In the first flask the generation time increased, in the second it decreased during cultivation.

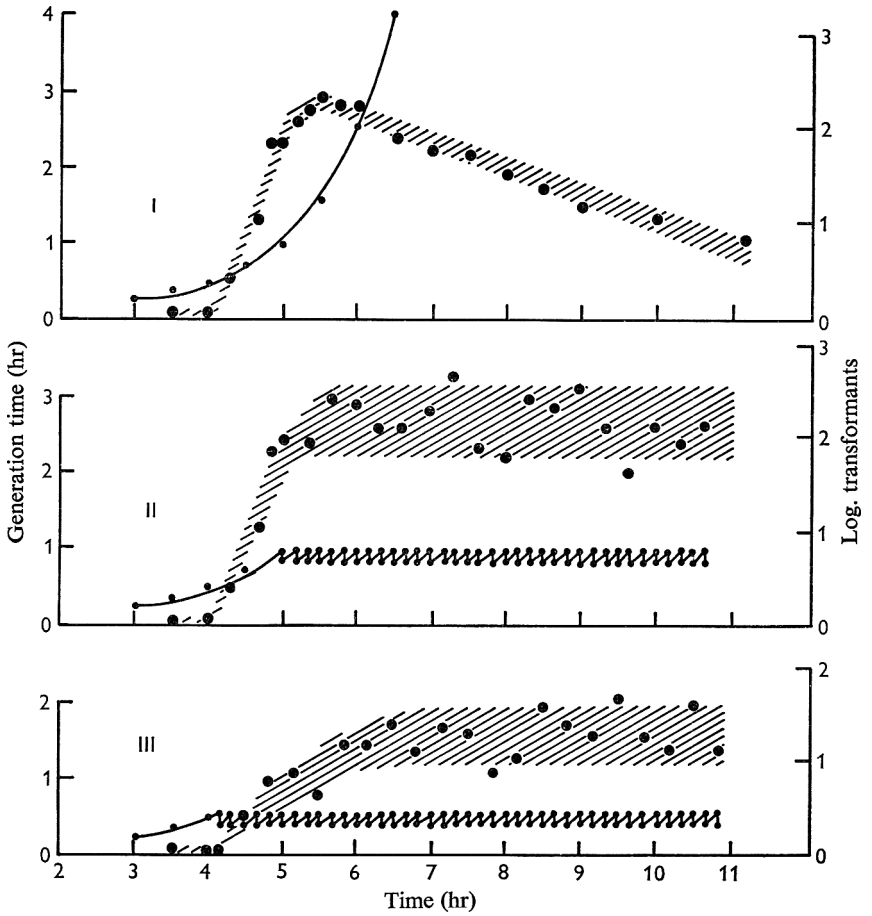

Fig. 2. Changes of competence in the balanced growth cultures. ●——●, Generation time. Shadowed area represents the competence.

Changes in competence in relation to the generation time are shown in Fig. 1.

In the first flask, competence followed the same course as in previous experiments (Horváth, 1967). In the second flask the curve of competence had a different shape because competence decreased for 1·5 hr, then increased for a short time, and finally decreased. It was easy to fix the peak of competence in the second flask by controlling the OD value of the bacterial suspension. At this time a small portion of the cell

suspension from the second flask was diluted with MGY medium to an OD of 0·1, and incubation continued. Competence was determined as before. The result was exactly the same as in the second flask.

These curves showed that competence depended on change in generation time. When the generation time increased or decreased from the optimal value, in both cases the competence decreased.

Changes of competence during balanced growth

Three bacterial suspensions were incubated in MGY liquid medium. In the first flask, the organisms multiplied in a closed culture. In the second flask balanced growth was maintained at a generation time of 50–55 min., and in the third the generation time was 25–30 min. During growth small portions of the cultures were taken and then

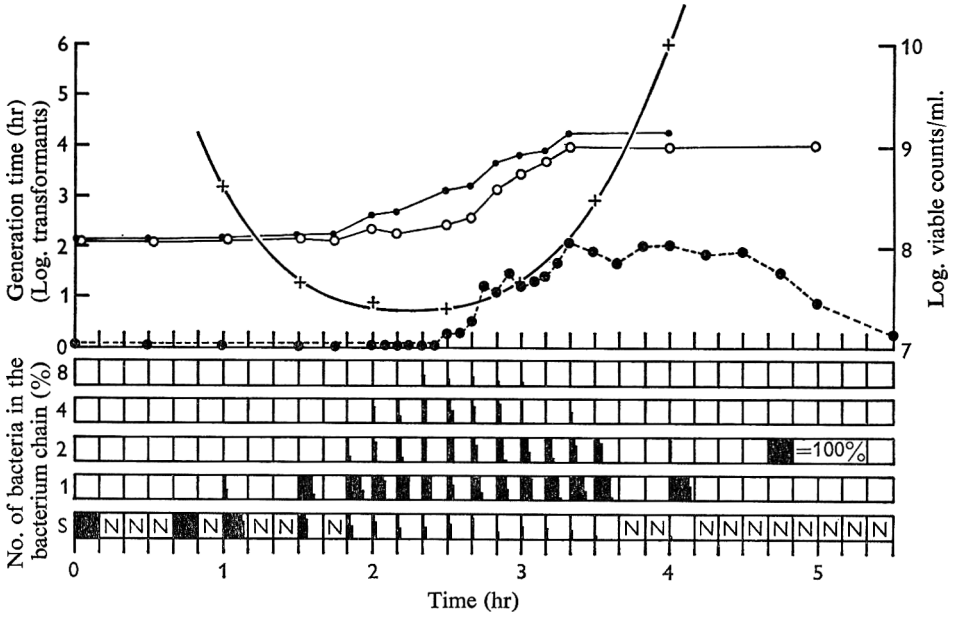

Fig. 3. Development of competence in MGY medium inoculated with spore suspension. Chain formation expressed in per cent. ●---●, Number of transformants; +——+, generation time; ○——○, uncorrected viable counts; ●——●, corrected viable counts. S = spores. N = not tested.

competence assayed. Figure 2 shows that competence increased or decreased as a function of the generation time. In balanced growth, competence remained at the same level during cultivation.

Relation between competence and cell division. It is an open question how the cell-division rate influences competence. To investigate this problem it is best to synchronize the culture from the time when competence appears (when the shortest generation time begins to increase). This is not easy to do and therefore a spore suspensions of *Bacillus subtilis* 168 *try*$^-$ was inoculated into MGY medium and incubated as usual. During growth, the competence, viable count, generation time and chain formation were determined (Fig. 3).

Figure 3 shows that competence developed when the generation time began to increase (after 2·5 hr), and its peak appeared after 3 hr 20 min.

Spores took a long time to germinate and so a synchronized culture could not be obtained. During growth the bacteria remained attached to each other after division and formed chains. After 2 hr 20 min., 5 % of the cells divided at least 3 times. During this period no competent bacilli were seen, but the generation time was then at a minimum. After 2·5 hr incubation cell division was asynchronous and we could not demonstrate a step like growth curve.

Viable counts were made on MGY agar. According to the uncorrected values, the bacteria began to multiply after 2·5 hr. When correction was made for chain formation, the corrected curve was obtained. According to this, the bacteria began to divide after 1 hr 45 min. incubation.

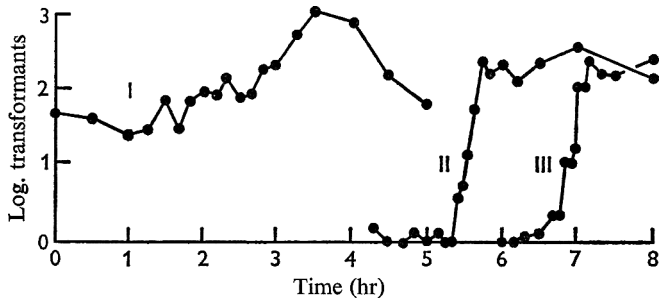

Fig. 4. Waves of competence in MGY media inoculated with different numbers of *B. subtilis* 168 *try*⁻. The inoculum size: I, $2{\cdot}95 \times 10^8$/ml.; II, $1{\cdot}18 \times 10^5$/ml.; III, $7{\cdot}45 \times 10^3$/ml.

Waves often appeared in the main curve of competence. In the following experiment three competence curves were determined at different occasions. MGY media were inoculated with different numbers of organisms and the number of transformants was determined from 5 replicate measurements (Fig. 4).

Figure 4 shows that the bigger the inoculum of bacteria, the bigger the waves of competence. When a small number of organisms was inoculated into the medium very small waves were seen. Three waves could be distinguished in curve I. The differences between the highest and lowest values of the waves were significant ($P < 0{\cdot}02$).

The waves were bigger when the MGY media contained less yeast extract or when minimal media were used.

An extremely marked wave of competence was demonstrated in the following experiment: $3{\cdot}6 \times 10^6$ bacilli/ml. were inoculated into MGY medium and grown only to 0·33 OD in order to keep the generation time short ($1{\cdot}9 \times 10^8$ bacteria/ml.). After centrifugation at 1600 *g* for 5 min., the packed organisms were resuspended in 5 ml. supernatant at 1·95 OD, and incubation continued until the OD rose to 2·4. During growth samples were taken for transformation to prototrophy and chain disruption was controlled (Fig. 5). During growth the bacteria divided very slowly. The short chains split up to give individual bacteria.

When a large number of organisms is inoculated into the medium cell division is nearly synchronous thereafter, it may be supposed that the waves were caused by the last cell division, before the development of sporulation.

Development and changes in competence under different conditions

The effect of differing amounts of yeast extract in MGY liquid media was tested. An overnight culture was used as an inoculum to give at the beginning of the experiment an OD value of 0·1. The numbers of transformants at the peaks of competence are shown in Fig. 6.

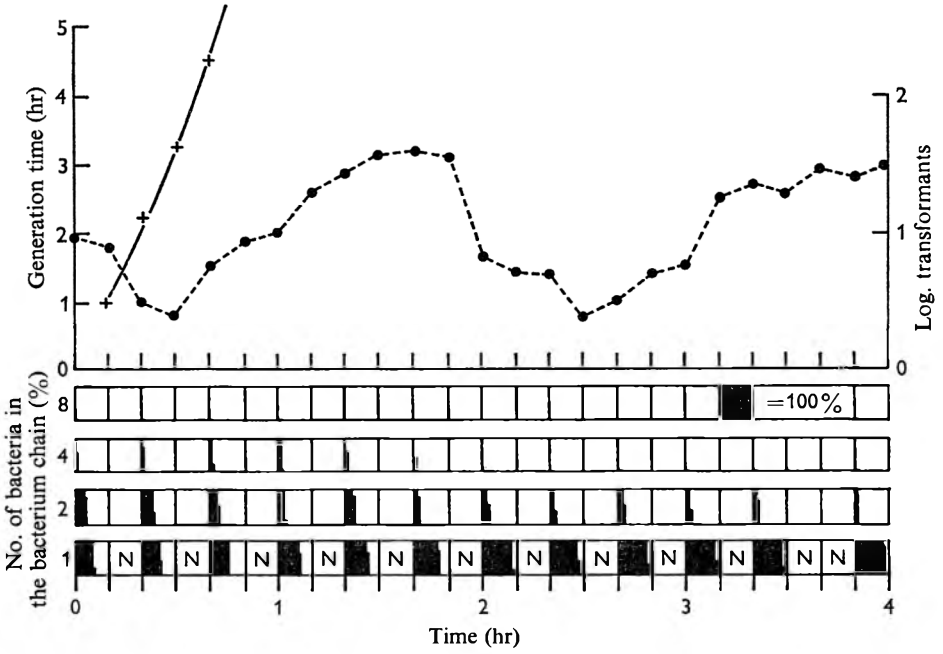

Fig. 5. Waves of competence in MGY medium inoculated with $1{\cdot}15 \times 10^9$ organisms/ml. of a well-grown *B. subtilus* 168 *try*⁻ suspension. ●---●, Number of transformants; +——+, generation time. N = not tested.

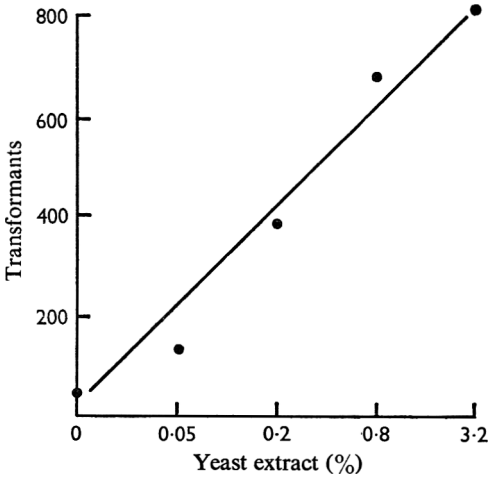

Fig. 6. Peaks of competence in MGY media containing different amounts of yeast extract.

Earlier the peak of competence was obtained with the smaller quantity of yeast extract. In Fig. 6 it is clearly seen that the peaks of competence are higher when larger amounts of yeast extract are used.

Bacillus subtilis 168 *try*$^-$ was inoculated into three flasks of MGY medium. The first was shaken during incubation, the second one shaken only for 3 hr, and the third one incubated without shaking. The results are shown in Fig. 7.

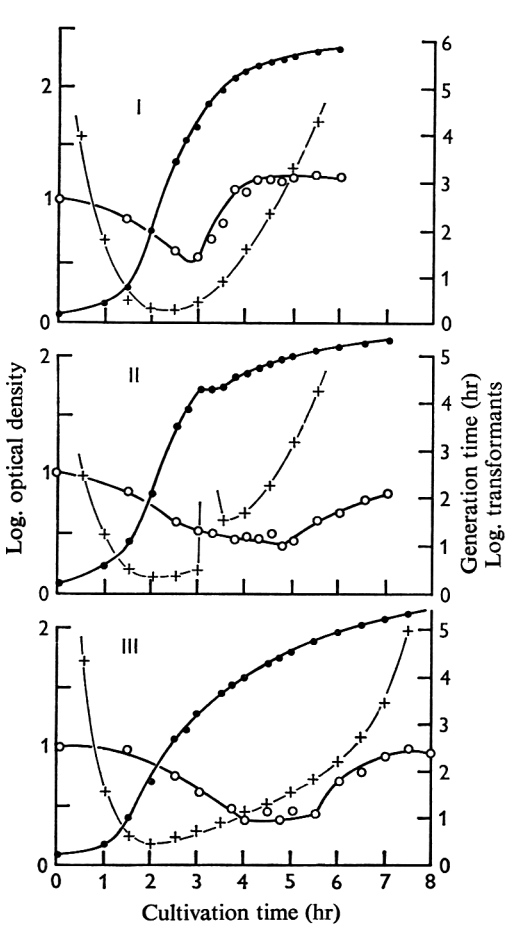

Fig. 7

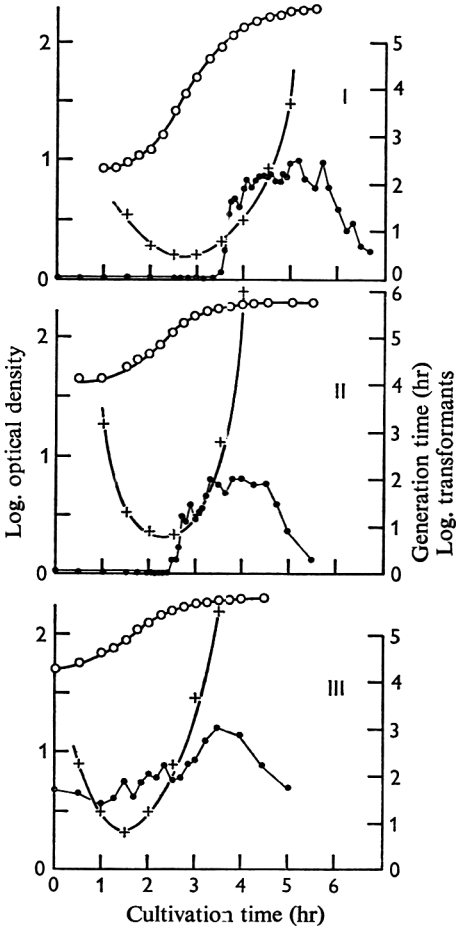

Fig. 8

Fig. 7. Development of competence in MGY medium in different amounts of oxygen. ○——○, competence; ●——●, optical density; +——+, generation time.

Fig. 8. Development of competence in MGY medium inoculated with spore suspension and precultivated organisms. ○——○, optical density; ●——●, competence; +——+, generation time.

In Fig. 7, I. Competence developed when the bacterial cells were shaken. During incubation competence decreased and after 3 hr increased quickly again up to a high degree.

In Fig. 7, II, shaking was stopped after 3 hr. For this reason competence could not develop as in Fig. 7, I. After an interval of 2 hr, competence rose slowly to a low level.

In Fig. 7, III, the low oxygen tension had a stimulatory effect on glycolysis (Pasteur reaction) serving to increase the efficiency of an anaerobic supply of energy. In this case competence decreased to a low level and increased again after 5 hr 30 min. The peak of competence was lower than in the first case.

When shaking the bacteria was stopped during the development of competence the competence began to decrease at once.

When the bacterial cells were shaken after 5 hr incubation, competence developed at once and its peak was the same as that found in the shaken culture.

Different quantities of spores (OD, 0·1 and 0·5) and 17 hr cultures (OD 0·5) of *Bacillus subtilis* 168 *try*⁻ were cultivated in MGY medium in the usual way. During growth samples were taken for the transformation (Fig. 8).

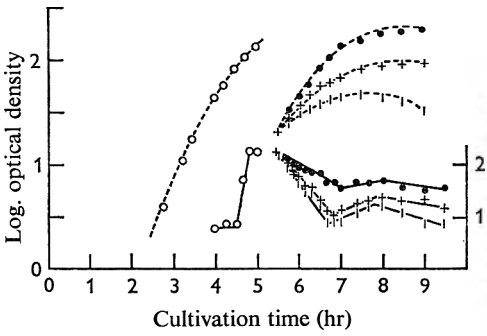

Fig. 9

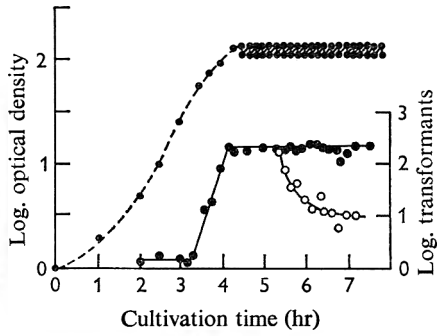

Fig. 10

Fig. 9. Changes of competence in media containing different compositions. – – –, Optical density; ——, competence; ●●●, values in medium 1; + + +, values in medium 2; | | |, values in medium 3; ○○○, values in MGY liquid medium.

Fig. 10. Changes of competence in balanced growth cultures at 37° and 0°. ●- - -●, Optical density; ●——●, the number of transformants at 37°; ○——○, the number of transformants at 0°.

With an initial count of $2{\cdot}46 \times 10^7$ spores/ml. competence developed after 3 hr 30 min., quickly rose, and then decreased gradually (Fig. 8, I).

In Fig. 8, II. the initial spore count was $1{\cdot}23 \times 10^8$/ml. Competence rose after 2 hr 30 min. but the peak was lower than in Fig. 8, I.

The inoculum of bacterial cells ($2{\cdot}95 \times 10^8$/ml.) was competent when they were taken from the agar slope, the peak of competence was high (Fig. 8. III).

The smaller the inoculum of spores, the later the time of the development of competence.

In the next experiment, bacteria were transferred from an overnight agar slope into MGY liquid medium to give an initial count of 10^6/ml. After 5 hr incubation the cells became highly competent and were then sedimented at 1600 *g* for 5 min. The packed organisms were resuspended in three media of the following compositions: (1) M/15 phosphate buffer (pH 7), 5 μg/ml. L-tryptophan, 0·5 % glucose, 0·1 % casein hydrolysate and 0·2 % yeast extract: (2) the first without yeast extract; (3) the second with 0·02 % casein hydrolysate.

The OD was adjusted to 0·2. The curves of competence determined by the usual procedure, using T-medium (Fig. 9).

The decrease of competence was lower in minimal medium than in the complete one.

In the following experiment bacteria were inoculated into 10 ml. MGY medium at an initial count of 5×10^6/ml. and incubated. When the bacteria showed maximal competence with a generation time of 55–60 min., balanced growth was initiated. A small portion of the organisms was taken after 5 hr 20 min. and put into an ice bath. The changes of competence were also followed in this bacterial suspension (Fig. 10).

When the organisms were kept at 0° the competence decreased by 1·1 log. units after 1 hr.

DISCUSSION

Genetic transformation by free DNA is possible only when the bacterial cells become competent. But, despite many reports, this property of bacteria has not been well defined (Ravin, 1956; Jensen & Haas, 1963; Spizizen, Reilly & Evans, 1966). The development of competence is influenced by both endogenous and exogenous factors which affect one another during growth.

One of the problems in the transformation procedure is that *Bacillus subtilis* strains which can be easily transformed contain organisms differing in competence so that the frequency of transformation is variable (Young & Spizizen, 1961). In *B. subtilis* 168 *try*$^-$, competence develops in late logarithmic growth when the bacteria multiply at a certain rate and it seems that this state may be associated with certain processes leading to sporulation (Young & Spizizen, 1961; Spizizen, 1965; Young, 1967). Asporogenous mutants of *B. subtilis* which remained competent have been isolated (Schaeffer, 1964).

In 1963 a lytic factor was demonstrated in the wall in those strains of *Bacillus subtilis* which can be easily transformed (Young & Spizizen, 1963; Young, Tipper & Strominger, 1964). In *B. subtilis* strains (168 *try*$^-$, 168 M, SB-25, etc.) we have also found that the good transformable strains had a high autolytic effect, while others which could hardly be transformed showed a very low lytic effect. We could not find any definitive proof that this factor (extract of the bacteria, or competent bacterial culture filtrate) facilitated the transformation.

Charpak & Dedonder (1965) demonstrated a substance in the supernatant of competent *Bacillus subtilis* 168 *try*$^-$ culture, and in the cell extract, which permitted transformation in a non-competent *B. subtilis* strain.

Exogenous factors, such as, the composition of the media, the temperature, and the inoculum size are also very important in the development of competence.

Many investigators use minimal media for transformation experiments (Anagnostopoulos & Spizizen, 1961; Young & Spizizen, 1961) but it was found here that if the bacteria are cultivated in MGY medium, the peak of competence is higher. There is no contradiction between these results. In a certain period of time competence is higher in a minimal medium and lower in a supplemented one; but the peak of competence is higher in the supplemented media when cultivation is continued. Competence also develops in MGY liquid media inoculated with spores of *Bacillus subtilis* 168 *try*$^-$ and in precultivated bacteria too. It also appears in bacteria cultivated on agar slope.

Other factors are also of importance. Aeration facilitates the development of competence, and when the supply of oxygen is decreased competence stops developing. In a previous paper (Horváth, 1967) we showed how the inoculum size influenced the

development of competence. The smaller the bacterial inoculum, the shorter the time required for the rise phase of competence

The OD value of a bacterial suspension is a valuable guide to competence although its optimal value depends upon the inoculum size (Horváth, 1967), the medium, the strains used in the experiment.

One of the important factors is the generation time which is determined by a lot of factors. The optimal generation time for competence varies according to the strains and the cultural conditions. Its average value is 45–60 min. which shows that most of the organisms are actively growing. In the pneumococcus transformation system, Tomasz (1965) also demonstrated that the competent cocci grew actively. Protein synthesis appears to be required for the maintenance of competence. Once organisms have an optimal level of competence, it is kept for some hours or decreases slowly. In this case the organisms might have a long generation time and it seems to be 'non-growth' competent bacteria.

Singh & Pitale (1967) could separate competent bacilli from actively dividing incompetent ones by zonal centrifugation of a culture of *Bacillus subtilis* in a sucrose gradient. Their results showed that competent bacilli were lighter than the incompetent.

In a closed culture competence appears when the growth medium becomes exhausted of some nutrients, and then increases very much faster than the growth rate of the bacteria. During cultivation, competence begins to decrease.

Competent bacteria are relatively resistant to penicillin, actinomycin D, and puromycin. They appear to be non-dividing organisms (Nester & Stocker, 1963; Nester, 1964; Singh & Pitale, 1967).

Hotchkiss (1954) obtained evidence in a pneumococcus transformation system that cell division played a role in the development of competence. With *Bacillus subtilis* no evidence was found that the division-synchrony of bacilli played such a role.

When the medium becomes exhausted the competence develops after the last cell division, and for that reason cell division plays a role in the development of competence. Because cell division is partly synchronous, waves of competence can be seen.

I should like to express my gratitude to Director Dr B. Györffy for his valuable instruction and to I. Kállay for her expert technical assistance.

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The Development of Radiation-resistant Cultures of *Escherichia coli* I by a Process of 'Growth-irradiation Cycles'

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(*Accepted for publication* 15 *September* 1967)

SUMMARY

The process whereby bacterial cultures are alternately grown and then irradiated (γ-radiation) through several 'cycles' has been studied as a means of developing radioresistant cultures of *Escherichia coli* I. The nature of the environment at the time of irradiation influenced the extent of development of radioresistance. Radioresistance development was higher when the bacteria were irradiated in an organic environment than in an inorganic environment. It is not thought that radioresistant mutants would be produced in high numbers by this type of process during a dose-fractionation procedure of food irradiation.

INTRODUCTION

The development of radiation-resistant strains of micro-organisms may play an important role in determining the effectiveness of the radiation-processing of foods. The work of Gunter & Kohn (1956) suggested that when a cell population consists of a mixture of comparatively sensitive and resistant cells, radiation can be expected to select the more resistant cells. Several authors have shown that radiation-resistance may be induced in microbial populations where radiation survivors are repeatedly grown and exposed to further doses of radiation. Erdman, Thatcher & MacQueen (1961) reported that specific bacteria of public-health significance could be induced to develop radiation-resistance by the repeated γ-irradiation of radiation survivors in cultures when using doses which destroyed a high proportion of the population. Erdman *et al.* (1961) developed radioresistance in *Escherichia coli* and *Streptococcus faecalis* cultures, but not in *Clostridium botulinum* type E or *Salmonella gallinarum*. Gaden & Henley (1953) obtained an increase in resistance of *E. coli* strains B and B/R by repeated γ-irradiation of survivors (although the same workers obtained an increased radiation-sensitivity with *E. coli* strain 15). Increased radioresistance, which was a stable character, was developed in *Saccharomyces cerevisiae* by Maisin, Lambert & van Duyse (1955) by repeated exposure of survivors of X-irradiation. Luckiesh & Knowles (1948) with *E. coli*, showed an increase in resistance to ultraviolet (u.v.) radiation among u.v.-irradiation survivors. Both Erdman *et al.* (1961) and Maisin *et al.* (1955) found that the development of radioresistance in cultures occurred in a stepwise fashion; and Erdman *et al.* (1961) and Gaden & Henley (1953) showed that during the course of resistance-development a plateau was attained beyond which no further increase in resistance to a constant dose could be obtained.

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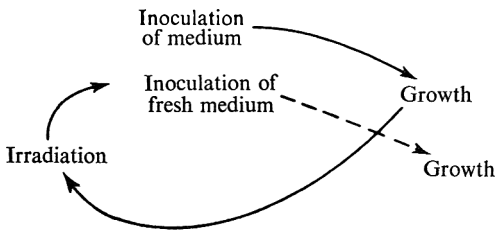
Erdman *et al.* (1961) showed that the radioresistance developed in cultures of *Streptococcus faecalis* and *Escherichia coli* was a 'true' radiation-resistance, and not a resistance to the possible toxic effects of reaction products from free radicals produced during the irradiation. Following the attainment of resistance-development plateaus, the *S. faecalis* and *E. coli* cultures were 1·6 times and 1·9 times more resistant than the respective parent cultures. Subsequently, Dr I. E. Erdman (1962; personal communication) showed that it was possible to extend the degree of radioresistance in such cultures by increasing the radiation doses at each plateau level. The development of the 'γ' series of radioresistant *E. coli* strains by Erdman (cited by Idziak & Thatcher, 1964) was based on this procedure, the dose being increased at each 'γ' level (or plateau) during multiple cycles of growth and irradiation to produce several distinct radioresistant cultures showing a progression in increased radioresistance. The details of the experimental procedure published by Erdman *et al.* (1961) were limited and were not in total agreement with those given in the personal communication from Dr I. E. Erdman. The purpose of the experiments described in the present paper was to establish a reproducible procedure for carrying out a series of growth-irradiation cycles on cultures of *E. coli*; and also to investigate the effect of growth medium and irradiation environment on radioresistance development. The work was not concerned with obtaining a maximal resistant development, but rather to compare radioresistance development in three well-defined series of experiments using the same radiation dose and the same limited number of cycles in each series. No attempt was made to reproduce the extreme radioresistance found by Erdman.

Growth-irradiation cycle. A growth-irradiation cycle is defined as the process initiated by the inoculation of the medium, the subsequent growth of the culture, the irradiation, followed by the immediate inoculation of fresh medium with the irradiated bacteria. It may be represented thus:



METHODS

Organisms. The test organism used for these studies was the strain of *Escherichia coli* 1 used by Erdman *et al.* (1961) and later by Dr I. E. Erdman (personal communication). This culture had been isolated from food, and was kindly supplied by Dr I. E. Erdman of the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada, in 1962. Stock cultures of the organism were maintained on TGY (Tryptone–glucose–yeast extract) agar slopes, stored at 4°, and subcultured monthly.

Growth media and culture method. Cultures were grown in 60 ml. medium in 500 ml. Erlenmeyer flasks incubated at 37°, with aeration provided by shaking the flasks on reciprocating-shaker water baths (The Mickle Laboratory Engineering Co., Gomshall, Surrey) at 100 oscillations/min. Growth media used were: (*a*) nutrient broth (Difco

Bacto) + 0·3 % (w/v) yeast extract (YE; Difco Bacto, dehydrated); (*b*) mineral salts medium, M 63, + 0·4 % (w/v) D-glucose (AnalaR). The composition of M 63 mineral salts medium (all chemicals AnalaR grade) was: KH_2PO_4, 0·1 M; $(NH_4)_2SO_4$, 0·02 M; $MgSO_4.7H_2O$, 0·001 M; $FeSO_4.7H_2O$, 0·00001 M, in de-ionized water, adjusted to pH 7·0 with 6 N-KOH. The above media were solidified, when required, by the addition of 1·5 % (w/v) Ion Agar No. 2 (Oxoid).

Colony-counting medium, Tryptone–glucose–yeast extract agar (TGY). Bacto tryptone (Difco) 5·0 g.; dehydrated Bacto yeast extract (Difco) 3·0 g.; D-glucose 1·0 g.; de-ionized water to 1 l.; pH 6·8.

Irradiation, and growth-irradiation cycle procedures. Gamma-radiation was provided by a ^{60}Co source. A laboratory unit (the 'Hotspot' Mk IV, U.K.A.E.A. design no. 0497) of nominal activity 1084 Ci. was supplied by the U.K.A.E.A.

Samples (30 ml.) of cultures grown to the stationary phase were irradiated in a specially constructed glass vessel. The vessel, 5 cm. diam. and 7·5 cm. high, was equipped with gassing inlet and outlet ports in the removable lid. Irradiations were performed at room temperature under aerobic conditions, oxygen (British Oxygen Co. Ltd.) being bubbled into the suspension before and during the irradiation at the rate of 0·4 l./min.

A dose of 15 krads was selected for use in the cycling procedure, and 1 ml. samples were removed aseptically from the irradiation vessel before and after the radiation dose was given. These samples were transferred to 60 ml. fresh medium, and the cultures (which became the control (c) and test (t) cultures) were incubated in the standard manner. The test culture had now undergone one growth-irradiation cycle. After incubation for 12 hr the irradiation process was repeated on the test culture and a 1 ml. sample removed to fresh medium; the control culture was also sampled into fresh medium. The cycling process was repeated until 7 growth-irradiation cycles had been completed. Cultures were stored on plates of the appropriate solid medium, and test and control cultures at this stage were designated t 7 and c 7, respectively.

It was found essential to adopt rigorous aseptic techniques throughout each series of cycles to avoid contamination of master cultures. All procedures, apart from irradiations, were done in a previously fumigated laboratory.

Estimation of growth. Growth was measured as the increase in extinction at 600 mμ by using a Unicam S.P. 600 spectrophotometer and 1 cm. glass cells, reading against a sterile medium blank. For growth estimations during the cycling procedures, duplicate cultures were used to eliminate the chance of contaminating master cultures of the control and test by repeated sampling during the incubation.

Radiosensitivity estimates, by colony counts. Suspensions of organisms were irradiated at a population density corresponding to an E_{600} of 1·5. Colony counts were made by the pour-plate method, with TGY agar as the recovery medium. Plates were incubated at 37° and colonies counted after 24 hr, and checked after further incubation. Counts were made in triplicate.

RESULTS

Growth-irradiation cycle procedures were designed in three series so that growth medium and irradiation environment could be varied.

Series A

Growth medium: nutrient broth+0·3% (w/v) yeast extract. Irradiation environment: bacteria irradiated in the growth medium.

Establishment of the radiation dose to be used during the process. Figure 1 shows the results of the radiosensitivity estimate made on 30 ml. of a culture in the stationary phase of growth. Survival (%) is plotted on a $\log_{10}$ scale against the dose. The D_{10} value (decimal reduction dose) for bacteria under these conditions was 4·8 krads,

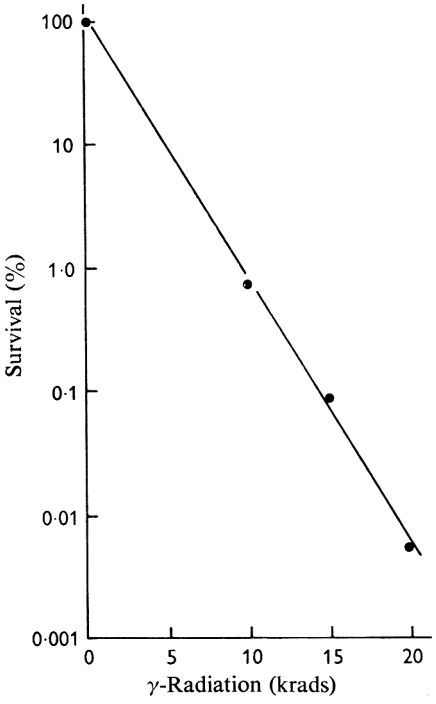


Fig. 1

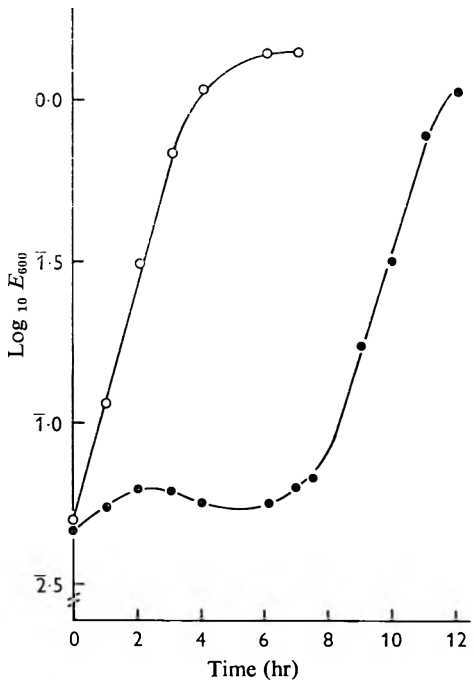


Fig. 2

Fig. 1. Dose–survival relationship for *Escherichia coli* 1 (the parent strain). Bacteria were grown to the final stationary phase, with shaking at 37°, in nutrient broth +0·3% (w/v) yeast extract, and irradiated in the growth medium. Survivors were estimated by the pour-plate method with TGY agar as recovery medium and incubated at 37° for 24 hr. Each point represents the mean value of counts in triplicate.

Fig. 2. Post-irradiation growth of the parent strain of *Escherichia coli* 1. Bacteria were grown to final stationary phase in nutrient broth+0·3% (w/v) yeast extract, then irradiated in the medium. Samples (1 ml.) were removed from unirradiated (control) and irradiated (15 krads) suspensions and transferred to 60 ml. nutrient broth+0·3% (w/v) yeast extract in 500 ml. Erlenmeyer flasks. Flasks were incubated, with shaking, at 37°. Samples were removed aseptically at intervals for E_{600} determination against a medium blank. ○, Control; ●, irradiated—15 krads.

and a dose of 15 krads inactivated slightly over 99·9% of the original population; this dose was selected as the radiation dose to be used during subsequent growth-irradiation cycle procedures. Erdman *et al.* (1961) irradiated cultures at doses giving over 99% mortality in parent cultures.

Figure 2 shows the effect of 15 krads on the bacteria under the conditions of series A, assessed by post-irradiation growth. This was done to establish at what time the bacteria again approached the stationary phase of growth so that they could be re-irradiated. After incubation for 12 hr bacteria surviving irradiation had passed through the exponential growth phase and approached the stationary phase. It was decided that cultures could be re-irradiated 12 hr after the initial irradiation and subculture. Throughout subsequent cycling procedures, irradiations were done after incubation for 12 hr.

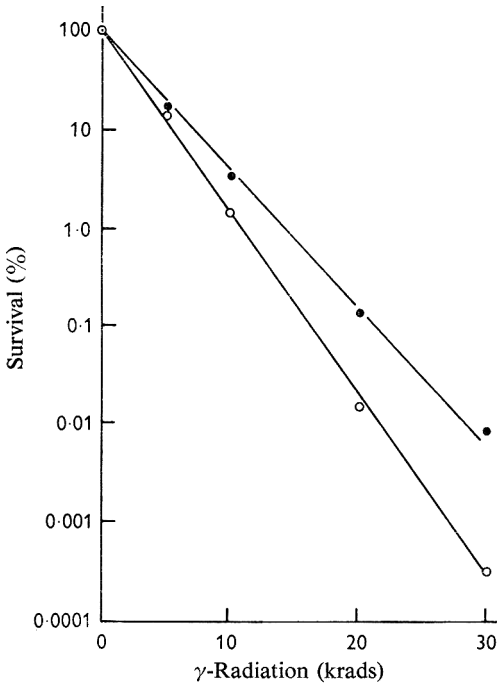


Fig. 3

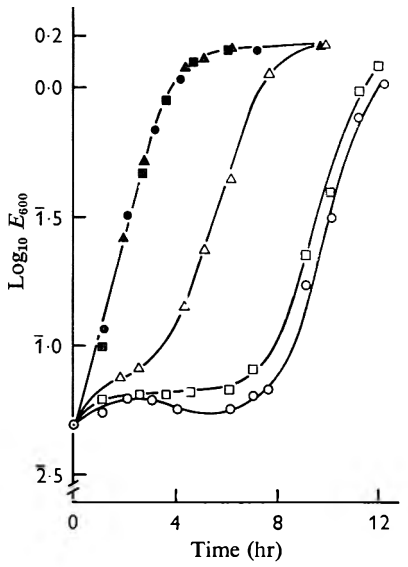


Fig. 4

Fig. 3. Dose–survival relationships for control and test cultures of *Escherichia coli* 1 following 7 complete growth-irradiation cycles (series A). Bacteria were grown to final stationary phase in nutrient broth+0·3% (w/v) yeast extract and irradiated in the growth medium. Survivors were estimated by the pour-plate method with TGY agar as recovery medium, incubated at 37° for 24 hr. Each point represents the mean value of counts in triplicate. ○, Control culture; ●, test culture.

Fig. 4. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as demonstrated by post-irradiation growth (series A). Samples (1 ml.) were removed from the cultures immediately following the 1st, 3rd and 7th irradiations (15 krads), and also from the unirradiated controls. Samples were transferred to 60 ml. nutrient broth+0·3% (w/v) yeast extract and flasks were incubated, with shaking, at 37°. During the incubation, samples were removed for the determination of E_{600} against a sterile medium blank. ○, ●, 1st cycle; □, ■, 3rd cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

Growth-irradiation cycle procedure in series A. The established growth-irradiation cycle procedure was done for 7 complete cycles. The control (c 7) and test (t 7) cultures were subcultured on TGY agar and stored at 4°. The two cultures were visually pure, and did not differ from each other in colonial or cellular morphology. The dose survival

curves in Fig. 3 show a marked development in radioresistance in the t 7 culture compared with the c 7 culture. D_{10} values were, respectively, 7·3 krads and 5·3 krads, and according to the difference in these values the increase in radioresistance was 38% after 7 cycles. The control D_{10} value was found to be increased from 4·8 to 5·3 krads following 7 cycles without irradiation. No real explanation can be suggested for this phenomenon, and the increase did not affect the result since % increase in radioresistance was derived from test and control D_{10} values after 7 complete cycles.

The progressive development of radioresistance with increasing number of cycles was also apparent in the post-irradiation growth curves plotted during various cycles. Figure 4 shows that while the curves for the test culture are gradually displaced in time towards the y axis with increasing number of cycles, the curves for control cultures are not displaced. This provides a clear indication of the development of resistance, for it is to be expected that the more resistant cultures will be less affected by the same dose of radiation, and hence grow more quickly than the initial irradiated culture. The method also indicates that the development of radioresistance is progressive (though irregular) and suggests a process of selection. The time required for the test culture to reach a $\log_{10} E_{600}$ value of $\bar{1}$·5 was 10 hr after the first irradiation, but only 5·5 hr after the seventh irradiation.

Series B

Growth medium: nutrient broth + 0·3% (w/v) yeast extract. Irradiation environment: M/15 phosphate buffer (pH 7·0).

This series of experiments was done as a control for series A, to determine the effect on radioresistance-development of irradiating bacteria in the presence of the organic medium and metabolic products. The experimental procedure was as in series A, except that before irradiation, the bacteria were harvested from the medium by centrifugation (6000 *g* for 10 min.), washed and suspended in the buffer to the original volume. Control cultures were similarly treated. Cultures were again taken through 7 complete cycles, and were then stored on TGY agar at 4°. Both c 7 and t 7 cultures appeared pure when examined by microscope, and were morphologically the same. D_{10} values calculated from the dose-survival curves for these cultures (Fig. 5) were 5·7 krads and 6·8 krads for the c 7 and t 7 cultures, respectively. The % increase in radioresistance according to these values was 19%. The post-irradiation growth curves obtained during cycling in this series (Fig. 6) are comparable to those obtained in series A (Fig. 4); however, according to colony-count estimations the development of resistance in series B was in fact only half that in series A for the same number of cycles. This appreciably lower development of resistance in series B suggests that in series A there was an effect of the medium which resulted in an enhancement of resistance development when the bacteria were irradiated in the presence of the medium or metabolic products.

Series C

This series of growth-irradiation cycles was made with a medium lacking yeast extract (YE), to determine whether or not this supplement had any effect on resistance development. Haas & Doudney (1957) showed that cultivation in the presence of YE could increase the mutational response of *Escherichia coli* to u.v. radiation. The medium also lacked nutrient broth.

Growth medium: mineral salts medium M 63+0·4% (w/v) glucose. Irradiation environment: bacteria irradiated in the growth medium.

Growth-irradiation cycles were done in the standard way, and after seven completed cycles c 7 and t 7 cultures were subcultured on medium M 63-glucose agar and stored at 4°.

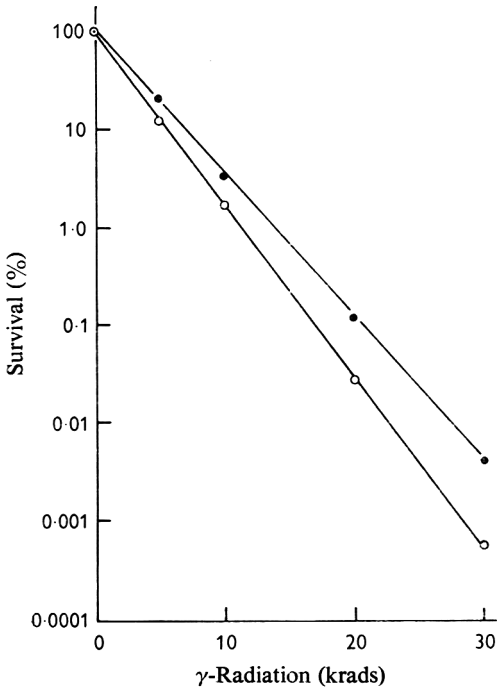


Fig. 5

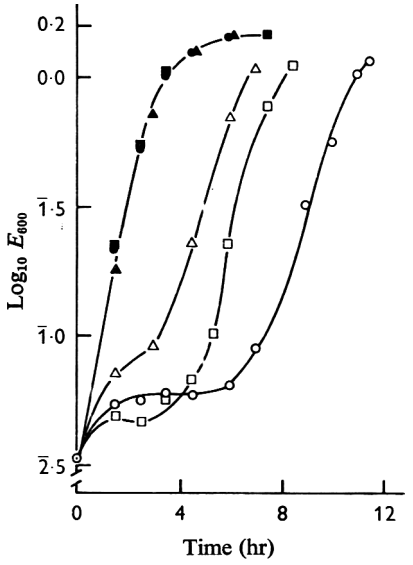


Fig. 6

Fig. 5. *Escherichia coli* 1. Dose–survival relationships for control and test cultures following 7 complete growth-irradiation cycles, (series B). Bacteria were grown to final stationary phase in nutrient broth+0·3% (w/v) yeast extract, harvested from the medium by centrifugation and resuspended to the same cell density in M/15 phosphate buffer (pH 7·0) before irradiation. Other details as in Fig. 3. ○, Control culture; ●, test culture.

Fig. 6. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as demonstrated by post-irradiation growth (series B). Samples (1 ml.) were removed from the bacterial suspensions in buffer immediately following irradiation (15 krads) in the 1st, 5th and 7th cycles. Samples were also taken from the corresponding unirradiated controls. The samples were transferred to 60 ml. nutrient broth+0·3% (w/v) yeast extract, which was incubated, with shaking, at 37°. Samples were removed at intervals during the incubation for determination of E_{600} against a sterile medium blank. ○, ●, 1st cycle; □, ■, 5th cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

Survival curves plotted in Fig. 7 show that D_{10} values for c 7 and t 7 cultures were 6·5 krads and 8·8 krads, respectively, representing an increase in radioresistance of 35%. Figure 8 illustrates the progressive development of radioresistance during the cycling procedure as seen by post-irradiation growth measurement. The survival curves for c 7 and t 7 cultures in Fig. 7 exhibit marked shoulders over the first part of the curves. The concentration of glucose in the growth medium was relatively high

(0·4% w/v), so the shoulders in both curves may represent the established glucose-effect (Hollaender, Stapleton & Martin, 1951).

DISCUSSION

The radiation doses used throughout the cycling procedures were comparatively high (initially inactivating over 99% of the population in each series), and it seems likely that the process of radioresistance development was one of selection rather

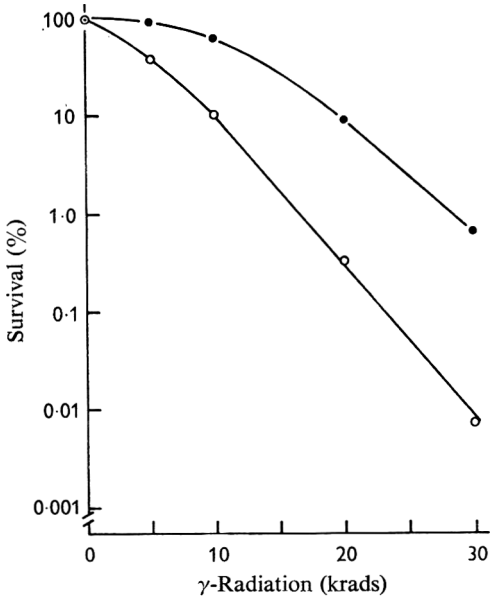


Fig. 7

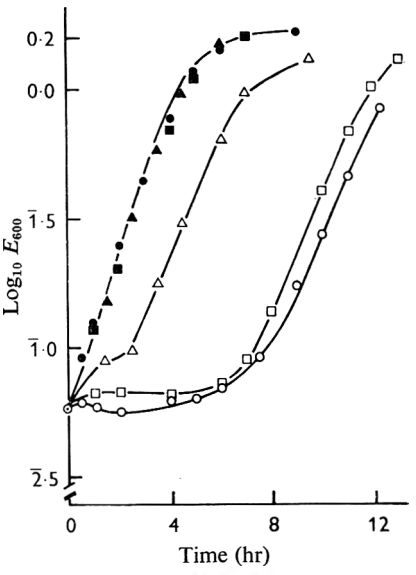


Fig. 8

Fig. 7. *Escherichia coli* 1. Dose–survival relationships for control and test cultures following 7 complete growth-irradiation cycles (series C). Bacteria were grown to final stationary phase in mineral salts medium M63+0·4% (w/v) glucose, and irradiated in the growth medium. Other details as in Fig. 3. ○, Control culture; ●, test culture.

Fig. 8. *Escherichia coli* 1. Development of radioresistance during the growth-irradiation cycle process, as shown by post-irradiation growth (series C). Samples (1 ml.) were removed from cultures in mineral salts medium M63+0·4% (w/v) glucose immediately following the 15 krads dose in the 1st, 3rd and 7th cycles. The samples were inoculated into 60 ml. fresh medium, which was incubated with shaking at 37°. Samples from corresponding unirradiated controls were similarly treated. E_{600} values were determined against sterile medium blanks at intervals during the incubation. ○, ●, 1st cycle; □, ■, 3rd cycle; △, ▲, 7th cycle. Open symbols, irradiated; closed symbols, unirradiated controls.

than one of progressive radio-adaptation. It is proposed that variations in experimental technique (particularly radiation doses, growth media, irradiation environment, growth phase of cultures) may partly explain the variable findings of other workers; and further that this explanation may allay scepticism about the process of repeated irradiation of survivors as a method of obtaining increased and stable radioresistance. The progressive development in radioresistance (particularly in series A and C) appears to be step-wise, though irregular. The post-irradiation growth curves obtained during various growth-irradiation cycles suggest that there might be a

threshold number of cycles after which resistance development becomes more rapid. This is indicated by the fact that growth curves in the 3rd cycle were not far removed from those of the 1st cycle, while the positions of the curves during the 7th cycle in these cases showed a very marked resistance development between the 3rd and 7th cycles. Post-irradiation growth curves provided further evidence for the development of radioresistance as, in each case, a dose of 15 krads in the 1st cycle caused a phase of post-irradiation cell lysis, whereas in the 7th cycle the bacteria were not affected to the extent that a lytic phase was observed. The effect of 15 krads in the 7th cycle was merely to introduce an apparent post-irradiation lag phase before exponential growth proceeded. The post-irradiation growth curves also suggest that a plateau in resistance development was being approached for the constant dose of 15 krads after 7 cycles. Post-irradiation growth curves after 7 cycles approach the control curves (representing the growth of unirradiated cells), and it is unlikely that under such experimental conditions (immediately following a dose of 15 krads) post-irradiation growth curves would show a displacement past the unirradiated control growth curves.

The varying reports in the literature on the radiosensitivity of a particular species of micro-organism can often be accounted for by variation in factors such as the strain of organism, growth phase, and conditions before, during, and after irradiation. However, the results presented in the present paper show that there was a definite increase in radioresistance in each test culture, and that the radioresistance developed was genuine as in each case the radioresistance of the test culture was compared with that of the corresponding control culture (taken through the same cycling procedure, without irradiation). Thus % increase in radioresistance in each series, obtained after a limited number of cycles, was shown for the experimental conditions of each series. Variations in conditions among experimental series were kept to a minimum by establishing, and adhering to, an experimental procedure which was repeated in each series. In this way any differences between each series of experiments were purposely designed, and thus differences in results from one series to the next were assumed to be due to imposed experimental differences.

The % increase in radioresistance following 7 growth-irradiation cycles was comparable in series C to that in series A. This indicates that the development of radioresistance in these experiments did not necessarily require the presence of yeast extract in the growth medium or irradiation environment. It would seem that the presence of organic metabolites and metabolic products, not necessarily from yeast extract, in the irradiation environment of series A and C may have influenced a greater development in radioresistance than in series B, where the irradiation environment was inorganic. This is supported by the fact that radioresistance developed in series A and C was approximately twice that developed in series B. The effect of enrichment of pre-irradiation culture media, particularly with glucose, leading to increased radioresistance has been widely reported (Hollaender *et al.* 1951; Stapleton & Engel, 1960). The results presented in the present paper, however, suggest that the nature of the environment at the time of irradiation becomes a contributing factor in radioresistance development by the process described. It is conceivable that the two organic environments (in series A and C), by virtue of their relative complexity, might be more mutagenic under irradiation than the inorganic buffer environment (series B).

The development of radioresistance by the growth-irradiation cycle process is

dependent on the growth of radiation survivors such that a large population density is reached before the next irradiation. The population attained might be expected to consist of bacteria representing a range in radiosensitivity. A progressive increase in radioresistance, obtained with increasing number of cycles, would require that this population should contain a high proportion of radioresistant bacteria which might be further selected by irradiation.

With the increased application of dose-fractionation processing in the irradiation of commercial goods, there arises the possibility that the repeated irradiation of the survivors of the initial dose might lead to the development of radiation-resistant micro-organisms through a mechanism analogous to that in the growth-irradiation cycle procedure. However, the results of the investigations reported in this paper suggest that it is unlikely that such a problem of resistance development will be encountered for two reasons: (*a*) development of resistance is dependent on several cycles: fractionation processes would rarely attain the number of irradiations warranted; (*b*) radioresistance development is dependent upon a substantial period of growth and multiplication of surviving bacteria between each irradiation. It is unlikely that a dose fractionation process would permit such growth of surviving bacteria, at least not to a high concentration, since inter-irradiation growth would require suitable conditions of temperature, pH value, nutrient supply and time. If dose-fractionation radiation-processing is applied to food products where it is conceivable that radioresistant development might be encouraged in radiation-survivors, particularly in unpackaged products, the danger could easily be averted by holding the products at low temperatures between successive radiation doses.

This work was done during the tenure of an A.E.R.E. contract (Agreement no. EMR/1200), and the authors wish to acknowledge the interest and co-operation of Mr F. J. Ley of the A.E.R.E. Wantage Laboratory.

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J. gen. Microbiol. (1968), **51**, 107–114
Printed in Great Britain

The Recombination of Bromouracil-containing Deoxyribonucleic Acid in *Salmonella typhimurium* Transduction

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(*Accepted for publication* 18 *September* 1967)

SUMMARY

Salmonella typhimurium tolerated the replacement of about 70 % of the thymine in the DNA by 5-bromouracil (BU). In phage PLT-22 transduction, the presence of BU in the donor DNA had no effect on the linkage relationship of two contransduced markers and did not conclusively alter the ratio of complete transductants to abortive transductants. The yield of transductants per plaque-forming unit was more than doubled by the treatment of the donor bacteria and phage with BU. However, more than half of the BU-treated phage particles, detected by electron microscopy, did not form plaques; when the multiplicity of infection was calculated from the titre of physical particles, the frequency of transductants was normal. It is concluded that recombination was not affected by the substantial incorporation of BU into the donor DNA. Treatment of the recipient bacteria with BU slightly modified recombination by decreasing the frequency of transductants by about 40 % and the linkage of the cotransduced markers by about 5 %. Because comparable results were not obtained when the donor DNA contained BU, it is suggested that these effects resulted indirectly from the treatment of the recipient bacteria.

INTRODUCTION

5-Bromouracil (BU) can be incorporated into bacterial DNA in place of thymine (Dunn & Smith, 1954). No consistent pattern of results has been established in studies investigating the effect of base-analogue substitution on genetic recombination in bacteria. Low concentrations of unifilar and bifilar BU-labeled DNA did not influence the efficiency of transformation in *Bacillus subtilis* (Szybalski *et al.* 1960; Gimlin *et al.* 1966) although earlier experiments suggested that the number of recombinants was depressed by treatment (Ephrati-Elizur & Zamenhof, 1959). In contrast, in *Escherichia coli* K 12 conjugation, the treatment of the Hfr parent with BU decreased the amount of the transmitted DNA which was integrated into the recombinant molecule (Folsome, 1960; Shchipkov & Samoylenko, 1966). The present paper, involving the *Salmonella typhimurium* phage PLT-22 transduction system, was designed to investigate further whether genetic recombination is affected by BU incorporation. The results show that recombination was apparently normal when the donor DNA contained BU but was slightly affected when the recipient bacteria had been treated with BU.

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METHODS

Organisms. The wild type and *leu-3 araB9* mutant (requiring leucine, unable to utilize arabinose) of the LT2 strain of *Salmonella typhimurium* were given by Dr P. F. Smith-Keary. The two markers are transduced on the same fragment by the temperate phage PLT-22. The *tryB4* mutant (requiring) tryptophan was obtained from the collection of Dr R. C. Clowes.

Media. Bacteria were grown in basal minimal medium M-26 and enriched M-26 (EM-26) medium, which were based on those of Folsome (1960). M-26 medium contained per litre: K_2HPO_4, 8·7 g.; KH_2PO_4, 6·8 g.; NH_4Cl, 1·0 g; Na_2SO_4, 0·1 g.; $MgCl_5$, 0·075 g.; $CaCl_2$, 1·5 mg.; $FeCl_3$, 1·2 mg.; thiamine, 1 mg.; glucose, 4·0 g. EM-26 contained, per litre, in addition: Ca pantothenate, 1 mg.; pyridoxine HCl., 1 mg.; thiamine, 1 mg.; uracil, 1 mg.; xanthine, 25 mg.; hypoxanthine, 25 mg.; Difco vitamin-free Casamino acids, 1 g. Plating media (Smith-Keary, 1960) included minimal medium enriched with 1 % (v/v) reconstituted Difco nutrient broth (EMM) and EMB arabinose agar. Enriched arabinose minimal medium (EAMM) had the same composition as EMM except that arabinose was substituted for glucose.

Preparation of bacteria. A sample (0·1 ml.) of a culture in medium M-26 was inoculated into 10 ml. of treatment medium composed of medium EM-26 supplemented with 100 μg./ml. of aminopterin (to render the bacteria thymine-requiring) + either thymine (Thy) or 5-bromouracil (BU) according to the treatment required. The culture was incubated with aeration for 12 hr at 37° before use in transduction.

Extent of 5-bromouracil incorporation. The extent of BU incorporation into the DNA was estimated as follows. The nucleic acids were isolated by the technique of Marmur (1961). The RNA was removed by hydrolysis with 0·5 N-NaOH, the DNA was hydrolysed in 72 % (w/v) perchloric acid and the bases separated by chromatography in isopropanol+hydrochloric acid (Dunn & Smith, 1954, 1957; Wyatt, 1951). BU and thymine run as a common spot in this solvent and the amounts of the two bases in the eluate were calculated by the method of Loring (1955) from the extinctions at 260 and 280 mμ.

Phage stocks and transduction. The procedures were based on those of Smith-Keary (1960). Phages were prepared by the soft-agar technique. The adsorption mixture was composed of equal volumes of a 12 hr Thy- or BU-containing wild type bacterial culture and a phage suspension of $1{\cdot}5 \times 10^7$ plaque-forming units (p.f.u.)/ml. The top layer agar and plate agar were made up of the relevant treatment medium (EM-26+aminopterin+Thy or BU) with 0·6 % and 1·2 % (w/v) agar, respectively.

For transduction experiments, the recipient *leu-39 araB9* bacteria were washed and resuspended in buffer at 3×10^9 bacteria/ml. Equal volumes of this and a phage suspension containing 3×10^{10} p.f.u./ml. were mixed, maintained at 37° for 9 min., and then plated on the selective medium. The number of bacteria surviving phage infection was assayed on EMM+leucine. After 48 hr at 37°, the Leu^+ and Ara^+ recombinants, selected on EMM and EAMM+leucine, were characterized for the second phenotype by replica plating on EMB arabinose and unenriched EAMM media, respectively.

Ultraviolet irradiation. The samples in buffer were exposed 35 cm. below a Hanovia 11 low-pressure mercury discharge tube. All manipulations were done in subdued lighting to avoid photoreactivation.

Preparation of electron micrographs. Electron micrographs of phage preparations

were kindly prepared by Dr D. Kay. The particles were negatively stained (Brenner & Horne, 1959) by mixing 0·7 ml. of undiluted phage preparation with an equal volume of 2 % (w/v) phosphotungstic acid (pH 6·2); 0·1 ml. of a suspension of polystyrene latex spheres of average diameter 880 Å (Dow Chemical Company) was added and the mixture sprayed on a grid. A series of electron micrographs was then prepared at a magnification of about × 45,500 by using a Philips 200 electron microscope. The latex spheres and phage particles, of head diameter about 470 Å (Bertani, 1958), were identified by their relative sizes and the numbers of two components thus determined.

RESULTS

The extent of 5-bromouracil incorporation

The base composition of the Thy- and BU-grown *leu-39 araB9* bacteria was estimated as 55·0 and 54·5 mole % of guanine + cytosine respectively (each value derived from two experiments). The extent of the replacement of thymine by BU was found to be 70 % in each of two experiments, demonstrating that the base analogue was extensively taken into the bacterial DNA. The growth rate of the bacteria in treatment medium was not affected by BU and the viability of the harvested 12 hr bacteria, estimated by comparing extinctions and colony-forming ability, was normal.

The incorporation of BU-DNA into the transducing particles in high-titre phage stocks, grown on BU-labelled bacteria in media containing BU, was investigated

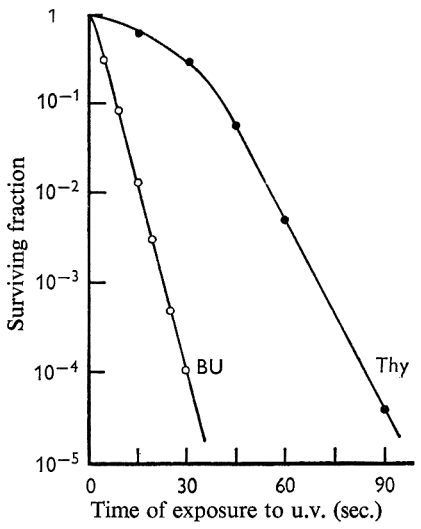

Fig. 1

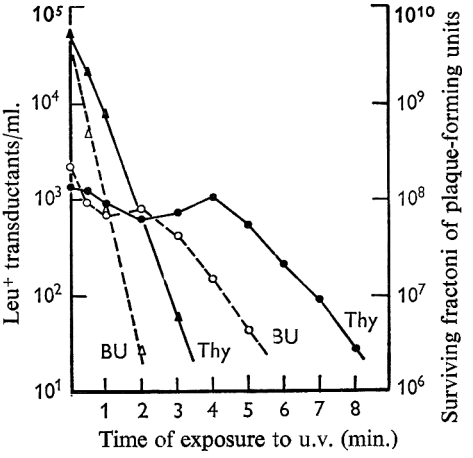

Fig. 2

Fig. 1. Survival after ultraviolet irradiation of the colony-forming ability of the *leu-39 araB9* mutant of *Salmonella typhimurium* which had been grown in the presence of either BU(○) or thymine (●). Similar survival curves were obtained for the wild-type strain.

Fig. 2. Inactivation by ultraviolet irradiation of the transduction potential (●, ○) and the phage infectivity (△, ▲) of a thymine (continuous lines) and BU (broken lines) PLT-22 phage preparation. The transduction mixture for each assay was composed of 0·4 ml. volumes of washed *Salmonella typhimurium leu-39 araB9* at $2{\cdot}5 \times 10^9$/ml. and irradiated phage of initial titre 5×10^9 p.f.u./ml. Values on the ordinates refer to Leu⁺ transductions/ml.transduction mixture, and the surviving fraction of p.f.u./ml. phage suspension.

indirectly. The presence of BU in DNA increases the sensitivity of bacteria to the lethal effects of ultraviolet radiation (Greer, 1960; Fig. 1). Thus, the kinetics of u.v.-inactivation of the transducing potential of Thy- and BU-treated phage were compared to examine whether the yield of transductants derived from the latter preparation was more effectively reduced by u.v. irradiation.

Each phage preparation was exposed to a series of doses of u.v. radiation. The ability of the phage to form Leu$^+$ transductants in the transduction *leu-39 araB9* (recipient) $\times \pm \pm$ (donor) was determined after each dose; the results are shown in Fig. 2. The more rapid loss of the transduction potential of the BU-treated phage lysate suggests that BU-DNA was included in the transducing particles. Similarly, the increased u.v. sensitivity of the plaque-forming units in this lysate suggests that the analogue was also incorporated into the phage DNA.

The frequency of transductants

The frequency of Leu$^+$ transductants arising in the transduction *leu-39 araB9* $\times \pm \pm$ was measured when one or both parents had been treated with BU. Although the yield of transductants can be influenced by the addition of small amounts of nutrient broth to the minimal plating medium (Witkin, 1956), the degree of enrichment in EMM, the standard plating medium, was found to be more than sufficient to allow the maximum expression of transductants in all of the experimental classes.

Table 1. *The numbers of Leu$^+$ transductants/10^8 bacteria surviving phage infection arising in the Salmonella typhimurium transduction leu-39 araB9* $\times \pm \pm$, *classed according to: 1a. the treatment of the recipient and donor bacteria with thymine or BU, and 1b. the treatment of the recipient bacteria and the multiplicity of infection.*

All multiplicities of infection (M.O.I.) are in terms of plaque-forming units per bacterium. Each number is the mean of values obtained in 3 or 4 replicate experiments.

	Treatment		Mean		No of experiments	
	Recipient	Donor	no.	S.E.		M.O.I.
1*a*	Thy	Thy	871	161	4	10
	Thy	BU	1837	214	3	10
	BU	Thy	514	14	4	10
	BU	BU	1110	138	3	10
1*b*	Thy	Thy	1401	93	3	10
	Thy	Thy	3112	353	3	27
	BU	Thy	863	135	3	10
	BU	Thy	1814	147	3	27

The estimated frequency of transductants for each of the four combinations of treatment, expressed as the number of Leu$^+$ transductants/10^8 bacteria surviving phage infection, is given in Table 1*a*. Two trends are apparent from the data. BU treatment of the recipient decreased the frequency of transductants by about 40 %. However, the frequency was more than doubled when BU-treated phage, grown on BU-containing bacteria, were used in the transduction. There is no evidence of any interaction between treatments in the BU $\times$ BU class.

The increase in transductants detected when the donor bacteria and phage had been treated with BU was further examined. The efficiency of the formation of a complete

transductant can be influenced at two stages: transfer of donor DNA into the recipient bacterium and the following process of its structural integration into the resident DNA. The efficiency of the second stage (genetic recombination) will be reflected in the ratio of complete transductants to abortive transductants (Ozeki, 1956), if these are the alternative fates of the transduced DNA. Thus it might be expected that the increased frequency of complete transductants would be associated with a compensatory decrease in the frequency of abortive transductants if the efficiency of recombination was increased when the donor DNA contained BU. Experiments were made to test this hypothesis.

To facilitate the detection of abortive transductants, the *tryB4* (recipient) × ± (donor) transduction system was used and the experimental procedure modified. The transduction mixture was composed of 1 ml. of an overnight broth culture of the recipient bacteria and 0·1 ml. phage suspension to give a multiplicity of infection of 0·7. Bacteria (4×10^7) were spread on each selective plate of minimal medium + 0·02 % (w/v) Difco vitamin-free Casamino acids. The results are shown in Table 2 and include the numbers of complete Try^+ transductants/10^9 phage particles for the two experimental classes involving the Thy- and BU-treated donor DNA and phage. All the complete and abortive transductants on a minimum of two plates in each experiment were scored and the ratio of the two types of transductant derived from the totals. The ratio for the BU-treated donor class was lower than that for the control but this probably reflected the fact that the abortive transductants were less easily identifiable. The total number of complete and abortive transductants/10^9 phage particles, calculated from these two sets of data, was 2734 for the control experiments and 4242 for the class involving the BU-treated donor bacteria. The lack of agreement between these totals suggests that the increased frequency of transductants, detected when the donor bacteria and phage had been grown in BU, primarily resulted from a raised frequency of genetic transfer and not from a greater efficiency of integration of BU-containing transduced DNA.

Table 2. *The ratio of complete to abortive Try^+ transductants and the number of complete Try^+ transductants/10^9 phage particles arising in the Salmonella typhimurium trasduction tryB4 × ±, classed according to the treatment of the donor bacteria with thymine or BU (multiplicity of infection: 0·7)*

Treatment		Ratio complete:abortive transductants	Complete transductants/10^9 phage particles
Recipient	Donor		
Broth	Thy	199:1211 (1:6·1)	385
Broth	BU	293:1470 (1:5·0)	707

A large proportion of the BU-grown phage did not produce plaques on indicator bacteria (see Dunn & Smith, 1954, 1957). If it be assumed that all the particles could adsorb to the bacteria and inject their DNA, the effective multiplicity of infection in transduction mixtures involving this component should have been calculated from the titre of particles and not that of plaque-forming units. Thus it is proposed that the increased frequency of transductants in the experiments involving BU donor material in Table 1*a* was caused by the multiplicity of infection being greater than the selected value of 10.

This hypothesis was examined with Thy- and BU-grown phage lysates prepared under parallel conditions and having titres of $4{\cdot}3 \times 10^{11}$ and $9{\cdot}0 \times 10^{10}$ p.f.u./ml., respectively. These preparations gave similar numbers of Leu$^+$ transductants/10^8 bacteria surviving infection to those given in Table 1*a*. The counts of particles were determined by electron microscopy. The ratio of particles to latex spheres for the Thy and BU preparations was 37:414 and 32:653, respectively. Therefore, the relative titre of particles in the BU-grown phage lysate was 0·55 that of the control preparation. If the latter had an efficiency of plating of about one, the titre of the BU-treated preparation can be estimated as $2{\cdot}4 \times 10^{11}$ phage particles/ml. Thus, it is argued that the effective multiplicity of infection in the BU-treated phage transductions in Table 1*a* approximated to 27 and not 10.

When the multiplicity of infection in the two transductions Thy and BU *leu-39 araB9* × Thy ± ± was raised from 10 to 27 p.f.u./bacterium, the numbers of Leu$^+$ transductants per 10^8 bacteria surviving infection were increased by about 115% (Table 1*b*). This increase corresponds with the trend established in Table 1*a*, and supports the concept that the treatment of the donor DNA with BU did not effectively alter the efficiency of transduction.

Table 3. *The percentage of the selected transductants jointly transduced for the second marker in the Salmonella typhimurium transduction leu-39 araB9* × ± ±, *classed according to the treatment of the parental strains with thymine or BU*

The total for each class was derived from the accumulated data of at least two replicate experiments. Each set of data, tested by a χ^2 test, was judged to be homogeneous (*P* always greater than 20%).

Treatment		Leu$^+$ selection		Ara$^+$ selection	
Recipient	Donor	Total Leu$^+$	% + +	Total Ara$^+$	% + +
Thy	Thy	1375	67·1	1591	71·3
Thy	BU	1707	67·0	1919	71·0
BU	Thy	2576	61·0	2930	66·4
BU	BU	1622	62·8	1776	65·2

The linkage relationship of cotransduced markers

The effect of BU treatments on recombination was also assessed from the linkage relationship of the two contransduced markers, *leu-39* and *araB9*. The results of these experiments are given in Table 3 and show the total numbers of the selected Leu$^+$ and Ara$^+$ transductants scored, and the percentages of these which were also recombinant for the unselected marker. The various percentages, compared by the normal approximation to the binomial distribution, show that linkage was normal when the transduced donor DNA contained BU, but was slightly decreased by about 5% when the recipient bacteria had been treated with BU (*P* ranging from 1·1% to 0·02%).

DISCUSSION

Transducing particles containing BU-substituted DNA were prepared by growing phage PLT-22 on BU-treated donor bacteria in media supplemented with BU. The presence of BU in the transduced DNA did not affect such parameters of recombination in transduction as the linkage of cotransduced markers and the ratio of complete

transductants (recombinants) to abortive transductants (non-integrated DNA). The frequency of transductants per plaque-forming unit was increased by the growth of the donor bacteria and phage in the presence of BU; but when non-viable phage particles detected by electron microscopy, were included in calculations of the multiplicity of infection, the yield of transductants was normal. Thus recombination was not affected when the transduced DNA contained BU. Although the transductions were carried out in media lacking BU, it is unlikely that the transduced fragments were replicated in the recipient bacteria prior to recombination, thus reducing the extent of BU substitution in the exogenotes. The following evidence suggests that the transduced DNA cannot be replicated as a fragment in the recipient bacterium. First, the transduced fragment in an abortive transductant is not integrated and it does not multiply (Ozeki, 1956). Secondly, fragments of bacterial DNA, transferred by conjugation and transformation, cannot multiply in the recipient bacteria if they are not part of a phage or sex factor (Jacob, Brenner & Cuzin, 1963). In generalised transduction by phage P1, a system which resembles that mediated by phage PLT-22, the transduced DNA is not associated with any phage DNA (Ikeda & Tomizawa, 1965).

BU treatment of the recipient bacteria decreased the frequency of transductants by 40 % and the linkage of two cotransduced markers by about 5 %. These effects, which were small and were not matched by comparable effects in transductions involving BU-containing donor DNA, most likely resulted indirectly from treatment which disturbed the physiology of the recipient bacteria in which recombination occurred. It is concluded that *Salmonella typhimurium* tolerated extensive BU incorporation and that the presence of BU in the DNA did not affect the molecular processes in recombination in phage PLT-22 transduction.

The extent of BU incorporation obtained in these experiments was greater than that achieved in investigations of the effect of BU on recombination in transformation and conjugation. The normal recombination of BU-containing DNA reported here is consistent with the observations that transformation in *Bacillus subtilis* was unaffected by the presence of BU in the transforming DNA, when the latter was used at concentrations which did not grossly exceed those required to saturate transformation (Szybalski *et al.* 1960; Gimlin *et al.* 1966). Thus conjugation in *Escherichia coli* K12 is the only bacterial genetic system in which BU-substituted donor DNA has been found to affect recombination; Folsome (1960) and Shchipkov & Samoylenko (1966) showed that, following BU treatment of the Hfr, the viable recombinants contained less of the transmitted DNA. Since *E. coli* is susceptible to growth retardation and killing when grown in BU (Dunn & Smith, 1954) and the genetic studies indicated that BU affected the viability of the Hfr and the kinetics of transfer of markers, the effect of BU on recombination in conjugation may reflect the sensitivity of the F^- bacteria to large segments of transmitted BU-containing Hfr DNA rather than a direct effect of the base analogue on the molecular interactions involved in recombination.

I am very grateful to Professor E. A. Bevan for his advice and encouragement during this work and thank Dr K. A. Stacey for interesting discussions. This investigation was partly supported by a Christopher Welch Scholarship from Oxford University.

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J. gen. Microbiol. (1968), **51**, 115–126
Printed in Great Britain

Studies on the Nature of the Killer Factor Produced by *Saccharomyces cerevisiae*

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(*Accepted for publication* 20 *September* 1967)

SUMMARY

Killer strains of yeast, *Saccharomyces cerevisiae*, liberate a killer factor into the medium which kills senstive yeast strains. The growth conditions necessary for the production of stable high-titre killer solutions and a biological assay for the killer factor are described. Purification was achieved by fractional precipitation with $(NH_4)_2SO_4$, dialysis, gel filtration and ultrafiltration. The fractionated killer factor is an unstable macromolecular protein which is inactivated by papain. The death of sensitive cells is not coincident with absorption of the killer factor, but can be delayed or prevented by variations in the environmental conditions. Sensitive cells are most susceptible to the action of the killer factor when in log phase. Treated resting cells on entering log phase are killed immediately.

INTRODUCTION

The killer reaction in yeast, *Saccharomyces cerevisiae*, was discovered by Bevan & Makower (1963), who described how certain strains could be classified into one of three phenotypes which they termed 'killer', 'sensitive', and 'neutral'. When killer and sensitive cells are grown together in the same culture medium, a high proportion of the latter are killed. Neutral cells neither kill sensitive cells nor are they killed by killer cells. Killing can occur without cell contact between killer and sensitive cells. The agent released by the killer cells which causes the death of the sensitive cells has been called the 'killer factor'. Studies described here show it to be a proteinaceous substance with a highly specific action spectrum, dependent on specific pH, temperature and aeration conditions.

The action of the killer factor resembles that of bacteriocins which do not cause lysis of the sensitive organisms and whose action is thus bactericidal, not bacteriolytic (Jacob & Wollman, 1959).

METHODS

Strains. Three haploid strains of *Saccharomyces cerevisiae* possessing the same mating type α, were used in the experiments: D1, a wild-type killer strain was used for the production of the killer factor; D2, a wild-type sensitive strain for the sensitive control experiments; and D3, a marked ad_1 mutant sensitive strain which produces a red pigment (Reaume & Tatum, 1949), as the sensitive strain in the tests performed to detect killing.

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Seeded agar phenotype test. The phenotypes of the strains were checked by replica plating (Lederberg & Lederberg, 1952) colonies derived from single cells onto buffered complete agar medium containing 0·003 % methylene blue and killer or sensitive cells (10^5 cells/ml.). Killer colonies were identified by zones of inhibition on the agar plates seeded with sensitive cells. Since methylene blue is a specific stain for dead yeast cells (Lindegren, 1949), the sensitive colonies were identified by a dark zone of dead cells on the agar plates seeded with killer cells. Neutral colonies showed no reaction on either killer or sensitive agar plates.

High-titre killer solutions. Stable high-titre killer solutions were obtained by incubating killer cells in buffered yeast complete or minimal medium (Bevan, 1955) at 22–24° for 3 days. The liquid minimal medium was supplemented with 0·05 % gelatine, and for agar media a 2 % (w/v) concentration of agar was utilized. All media were buffered at pH 4·8 with a 0·077M citrate/phosphate buffer (Gomori, 1959). The solutions were sterilized by filtration through Oxoid membrane filters

Well test. The Wilkins technique (1949) for obtaining inhibition zones in seeded agar was used for the assay of the killer factor in liquid media. 0·05–0·1 ml. aliquots of the solutions to be assayed were added to 4 holes 13 mm. diam. in 10 ml. seeded agar plates (10^3 ad_1 sensitive cells/ml.). The plates were kept for 0–2 hr at room temperature before being incubated overnight at 28°.

Agar-layer technique. The technique of assaying for the killer factor in agar medium was evolved by Makower (1964). Plates spread with a lawn of wild-type killer or sensitive (control) cells were incubated for 5–7 hr at 28° before a 10 ml. cooled agar layer was placed on top of the background cells. The plates were then incubated for 6–9 hr before spreading the marked (ad_1 red) sensitive cells (150 cells/plate) on the surface. After a further 18 hr incubation the plates were replicated onto fresh agar medium, and the % of marked sensitive cells killed was obtained by comparing the number of red colonies arising on treated (killer background) and control (sensitive background) plates.

Purification. The killer factor was precipitated from minimal medium solutions supplemented with 0·05 % (w/v) gelatine by 30 % $(NH_4)_2SO_4$ saturation. The sediments were washed and resuspended in 0·077M-citrate/phosphate buffer at pH 4·8 before being dialysed against the buffer. The killer factor was further fractionated by gel filtration at 0–4° with 1·5 × 30 cm Sephadex G-150 gel columns prepared by the method of Andrews (1965). The columns were equilibrated and eluted at a flow rate of 20 ml./hr with 0·0853 M-citrate/phosphate buffer (pH 4·8) containing 0·008M-$MgSO_4$: 2 ml. starting samples were applied to the columns, and the effluents collected in 5 ml. fractions. The effluent volume containing the purified killer factor was concentrated by the pressure dialysis method of Hofsten & Falkbring (1960), using Visking tubing 8/32 at 0–4°.

Examination of column effluents. The killer factor was assayed by the Well test; and the proteins and nucleic acids by light-extinction measurements at 280 and 260 mμ. Proteins were also measured by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955).

Sucrose density-gradient centrifugation. 0·5 ml. killer factor samples were layered on a 4–18 % sucrose gradient and the tubes centrifuged at 107,000 *g* for 6 hr at 0–4° in an M.S.E. Super 40 swing-out rotor. The contents of the tubes were collected and assayed as 8–10 drop fractions after the bottoms of the tubes had been punctured.

Agar-gel electrophoresis. The electrophoresis was carried out using microscope slides covered by a buffered homogeneous 1 % (w/v) Oxoid Ionagar layer 2 mm. in thickness, at a constant current of 2 mA per inch width of agar and at a potential of 30 V for 15–20 hr. After the electrophoresis the agar slides were cut in half longitudinally and each half was characterized for proteins and killer activity respectively. Proteins were stained by the nigrosin method of Weime (1965). The killer factor was identified by pipetting in a line down the centre of the agar strip, 0·075 ml. of a fourfold-strength buffered complete medium containing 0·06 % methylene blue and 10^4 sensitive cells/ml. After overnight incubation at 28° the slides were examined for zones of inhibition.

Electron Microscopy. Serial dilutions of the fractionated killer factor were shadowed with chromium and then examined under a Siemens Elmiskop 1 A electron microscope.

RESULTS

Well-test assay technique. Although subject to the effect of well-known variables like duration of incubation (Kavanagh, 1963), type of medium or genotype of the seeded sensitive cells (Woods, 1966), the Well test under standardized conditions was a reliable quantitative assay for the killer factor. The results in Fig. 1 show that at high concentrations of the killer factor there was a linear relationship between the square of the diameter of the inhibition zone and the log of the killer factor concentration.

Effects of physical and chemical agents on the production and stability of the killer factor

Effect of pH. The optimum pH for the production and stability of the killer factor was determined by incubating killer cells at 22° for 2 days in media buffered at different pH values between 3·0 and 5·4. The cells were removed by filtration and the filtrates retained at 28°, 22°, 12° and 4° for 1 hr and 24 hr, before the killer activity was estimated by the Well test.

The results shown in Table 1 indicate that the optimum pH for the production and stability of the killer factor in liquid medium lies within the narrow range pH 4·6–4·8.

Effect of temperature. To determine the effect of temperature on the killer factor in liquid medium, aliquots of a killer solution were kept at 18°, 23°, 25°, 28° and 37° respectively for different time intervals between 0 and 4 hr before estimating the killer activity by the Well test.

The results expressed in Fig. 2 illustrate that in buffered liquid medium the killer factor was inactivated by temperatures above 25°. Such inactivation may be due to a protease derived from the yeast cells and the temperature effect may reflect the Q_{10} for digestion.

In agar medium, on the other hand, the killer factor was markedly more stable and was only inactivated by temperatures above 42° (Fig. 3). This was shown using the agar-layer technique and comparing the percentage killing on killer plates incubated at 28°, 37°, 42°, 43°, 44° and 45° respectively for 5 hr after the addition of the top agar layers and also an initial incubation period for 7 hr at 28°. Makower (1964) showed that the length of this initial period at 28° between the addition of the top agar layers and the spreading of the sensitive cells greatly affected the percentage killing. Thus, a decrease in the percentage killing between the killer plates incubated throughout the experiment at 28° and those incubated at different temperatures following the initial

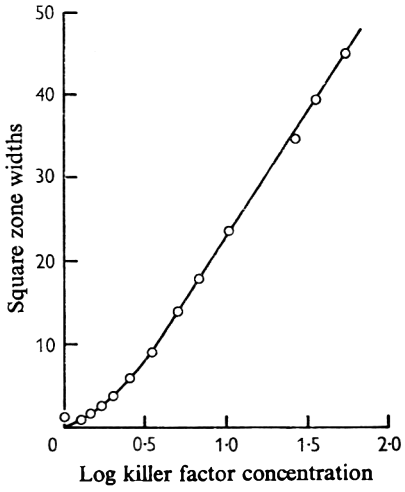

Fig. 1

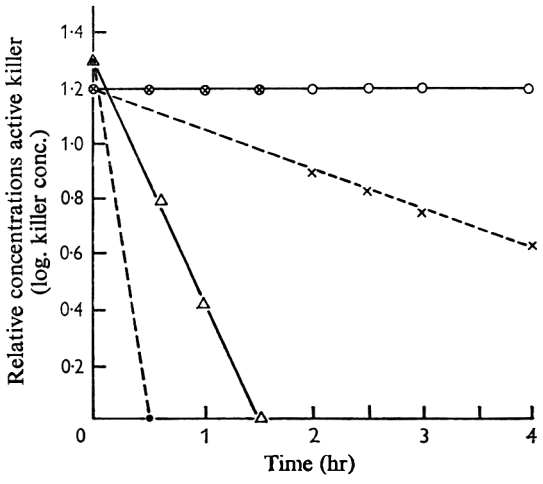

Fig. 2

Fig. 1. Dilution of the killer factor: Well test.

Fig. 2. Effect of temperature on the killer factor in liquid complete medium. ○, 18° and 23°; ×, 25°; △, 28°; ●, 38°.

Table 1. *Effect of pH on the stability of the killer factor*

	Relative concentrations of active killer (log killer concentrations)				
pH of medium	0 hr (cultures harvested)	1 hr 28°	24 hr 22°	24 hr 12°	24 hr 4°
5·4	0	0	0	0	0
5·2	0	0	0	0	0
5·0	0·53	0	0·53	0·53	0·53
4·8	1·40	0·53	1·40	1·40	1·40
4·6	1·40	0·53	1·40	1·40	1·40
4·4	1·40	0	0·75	1·40	1·40
4·0	1·40	0	0	1·40	1·40
3·6	0·53	0	0	0·10	0·53
3·4	0·30	0	0	0	0·30
3·2	0	0	0	0	0
3·0	0	0	0	0	0

incubation period, would indicate that the killer factor produced at 28° was being inactivated.

It can be concluded from these experiments that the inactivation of the killer factor by temperature depends upon the composition of the medium.

Surface inactivation. It was observed that the killer activity of a minimal medium solution disappeared on sterilization by filtration. To determine whether or not the inactivation was due to surface inactivation caused by aeration, the effect of vigorously shaking aliquots of killer complete medium and minimal medium containing increasing quantities of gelatine for 0, 2, 4 and 8 min. at 22° was studied.

It can be seen from Fig. 4 that the killer factor was rapidly inactivated when

vigorously aerated and, like many physiologically active proteins and bacteriophages, this surface inactivation was prevented by gelatine (Adams, 1948).

Effect of yeast extract. The results in Table 2 show that yeast extract enhanced the production of the factor by killer cells. A similar effect of yeast extract on the production of maltases by *Saccharomyces oviformis* was reported by Lewis (personal communication).

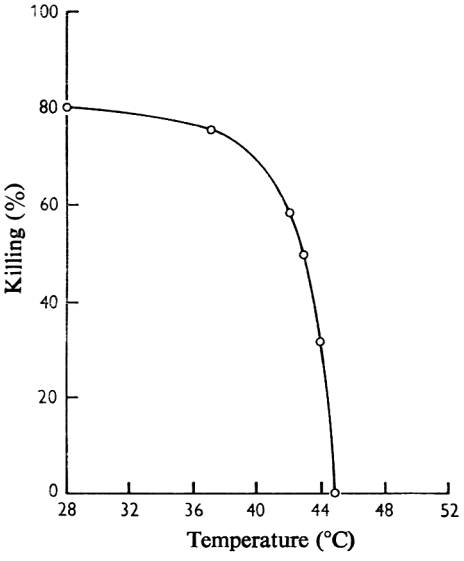

Fig. 3

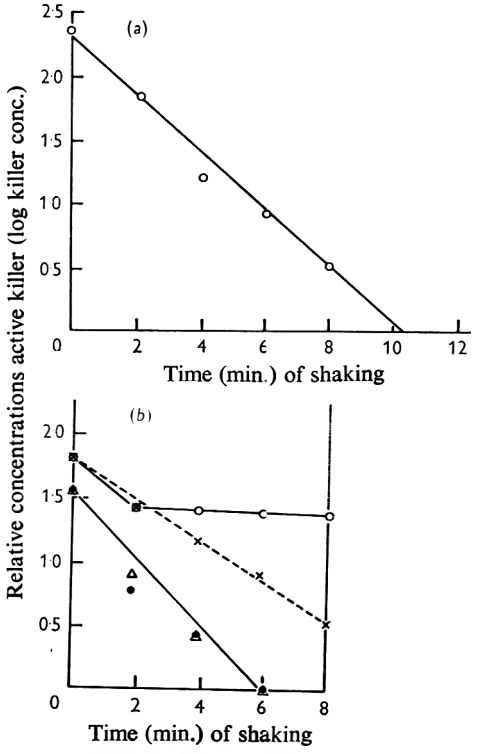

Fig. 4

Fig. 3. Effect of temperature on the killer factor in agar complete medium.

Fig. 4. Effect of vigorous aeration on the killer factor. (*a*) Inactivation of the killer factor in complete medium by shaking. (*b*) Inactivation of the killer factor in minimal medium by shaking in the presence of increasing quantities of gelatine. ○, 0·25 % (w/v); ×, 0·05 % (w/v); △, 0·005 % (w/v); ●, 0 % (w/v) gelatine.

Table 2. *Effect of various growth media on the production of the killer factor by killer cells*

Type of killer medium	No. of yeast/ml. at harvesting	Relative concentrations of active killer (log killer concentration)
CM* + gelatine	14×10^7	2·3
MM† + gelatine + yeast extract	12×10^7	2·3
MM + gelatine + peptone	14×10^7	1·59
MM + gelatine + casein hydrolysate	$14{\cdot}5 \times 10^7$	1·40
MM + gelatine	10×10^7	1·05

* Complete medium. † Minimal medium.

PURIFICATION

$(NH_4)_2SO_4$ fractionation. The results of the fractionation of the killer factor from chemically defined media, summarised in Table 3, indicate that it was precipitated by 70–81 % saturation with $(NH_4)_2SO_4$ from complete and minimal media. However, when 0·05 % (w/v) gelatine was added to the minimal medium the factor was precipitated with the gelatine at 25–30 % saturation with $(NH_4)_2SO_4$ and yielded a stable killer sediment with the highest specific activity. Agar-gel electrophoresis studies confirmed that only the killer factor and gelatine were precipitated (Fig. 6). The results

Table 3. *Fractionation of the killer factor by $(NH_4)_2SO_4$ from complete and minimal media*

Killer factor solution	% saturation with $(NH_4)_2SO_4$ required to precipitate killer factor	Stability resuspended sediment		Specific activity sediments in buffer
		Complete medium	Buffer	
Killer factor + complete medium	70–81	+	+	100
Killer factor + minimal medium	70–81	+	−	0
Killer factor + minimal medium + 0·05 % (w/v) gelatine	25–30	+	+	850

+, Stable. −, Unstable.

Table 4. *Concentration by ultrafiltration of the purified-killer-factor peak and a sensitive control sample (effluent volume 75–100 ml.) after gel filtration with Sephadex G-150*

Samples	Relative concentrations of active killer	Absorption		Folin test: absorption
		280 μ	260 μ	750 μ
Killer *a*	0·10	0·015	0·025	0·01
Killer *b*	0·53	0·155	0·1875	0·03
Killer *c*	0	−0·005	0	0·0125
Sensitive *a*	0	−0·02	−0·03	0·0075
Sensitive *b*	0	−0·015	−0·02	0·0075
Sensitive *c*	0	−0·025	−0·0375	0·0075

a, Effluent volume 75–100 ml. before concentration by ultrafiltration.
b, Sample concentrated by ultrafiltration.
c, Buffer removed during ultrafiltration.

also show that the fractionated killer factor was not stable but could be stabilized by resuspension in a diluent containing gelatine or the other proteins present in complete medium.

Gel filtration. The gel-filtration elution profiles of the dialysed killer factor plus gelatine (Fig. 5) illustrated that the factor was eluted at two peaks: a sharp peak between 15 and 30 ml. effluent volume coinciding with the gelatine peak which was excluded by Sephadex G-150 and was eluted at the void volume; and a second fractionated peak eluted between 70 and 110 ml. The fractionated killer factor was unstable and became inactive after retention at 0–4° for 34 hr. It can also be seen from Fig. 5 that there was no correlation between the second killer peak and the absorption at 260 and 280 mμ. The considerable degree of killer activity of this fraction as indi-

cated by the Well test illustrated the high sensitivity of this biological assay as compared with the physical and chemical methods employed. It was found possible to estimate the killer factor, by physical and chemical tests, only after the fractionated factor had been concentrated by ultrafiltration (Table 4).

The elution of the killer factor in two peaks suggested that either there were two different factors, or polymers capable of causing the killer reaction. Alternatively the fractionation by gel filtration resulted in the separation of only some of the killer

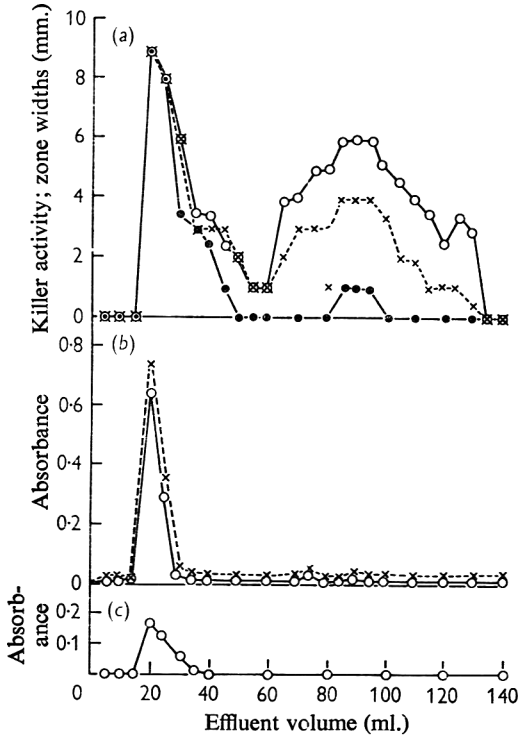

Fig. 5

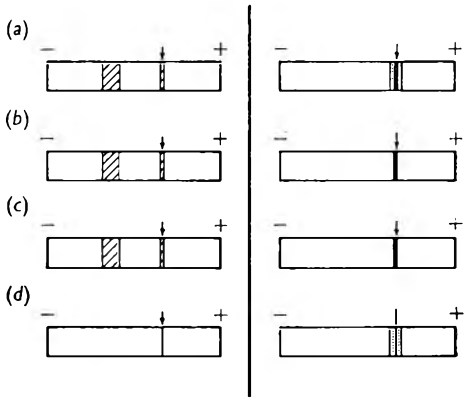

Fig. 6

Fig. 5. Elution profiles of the killer factor and gelatine sample after gel filtration with Sephadex G-150. (*a*) Killer activity at different time intervals between 0 and 34 hr. after gel filtration. ○, 0 hr; ×, 12 hr; ●, 34 hr. (*b*) Absorption at (×) 260 mμ and (○) 280 mμ. (*c*) Folin–Ciocalteu test for proteins (absorption at 750 mμ).

Fig. 6. Agar-gel electrophoresis. (*a*) Killer factor+gelatine sample after 30% $(NH_4)_2SO_4$ fractionation. (*b*) Sensitive control solution + gelatine sample after 30% $(NH_4)_2SO_4$ fractionation. (*c*) Gelatine sample. (*d*) Purified killer factor sample after concentration by ultrafiltration. ↓, origin, ▨, protein stain (gelatine); ■, purple stain (gelatine at origin + methylene blue); ▦, zone of inhibition (killer factor).

factor from the gelatine. To distinguish between these possibilities an attempt was made to separate the killer factor and gelatine by sucrose density-gradient centrifugation prior to gel filtration. Although the killer factor was dispersed throughout the gradient (Woods, 1966), it was possible to separate the bulk of it from the gelatine, since the latter was confined to a sharp peak at the top of the gradient.

After gel filtration this gelatine-free factor was eluted between 65 and 100 ml. effluent volume only. It was therefore concluded that the killer factor eluted at the

void volume was attached to the gelatine, and was thus not fractionated by gel filtration with Sephadex G-150.

It is interesting to note that the killer factor was hetero-dispersed after centrifugation in a sucrose gradient and also following ultracentrifugation in complete medium for 3 hr at 107,000 *g*.

The killer factor and gelatine sample eluted at the void volume were recycled but the killer factor was again only eluted at the void volume along with the gelatine. Recycling the fractionated killer factor failed to yield an effluent with any killer activity, presumably due to its instability in the absence of gelatine and to dilution effects.

In fractionations by gel filtration where the separation is governed entirely by the molecular-sieve effect, the distribution coefficient (*Kd*) cannot be greater than unity, and it is possible to estimate the molecular weights of proteins from the elution volume (Granath & Flodin, 1961; Andrews, 1965). However, the *Kd* for the separation of the killer factor was 2·59. This indicates that adsorption was also taking place, and it was therefore impossible to estimate the molecular weight of the killer factor by this method.

Agar-gel electrophoresis. This technique was used to demonstrate the fractionation of the killer factor during the purification process. The pherograms of the experiments illustrated in Fig. 6 show that the killer factor remained at the origin and was not affected by electrophoresis at pH 4·6. The purified killer sample, which contained no detectable contaminant proteins, was only identifiable by a zone of inhibition at the origin. The dialysed killer sample, which had not been further purified, contained only the killer factor and gelatine (Fig. 6).

Electrophoresis was carried out at different pH values between pH 4·0 and 5·0 and several conclusions can be made from the results. First, the killer factor was only identifiable by zones of inhibition at the origin between pH 4·6 and 4·8; this was expected as it is unstable at other pH values. Secondly, there was no other migrating protein band at any of the pH values besides that formed by gelatine.

Degree of purification. The results given in Table 4 show that a 40-fold purification of the killer factor was achieved by the procedures described. It should be pointed out, however, that this estimate is a conservative one due to the fact that the killer factor was gradually inactivated once it had separated from the stabilizing gelatine. Thus a certain amount of inactivation always occurred before the assay procedure could be performed.

Electron microscopy. Serial dilutions of the purified killer factor were examined under the electron microscope but it was not detected by the technique adopted.

Effect of papain. Evidence for the proteinaceous nature of the killer factor was obtained by the study of the effect of the plant protease enzyme, papain, which has a maximum enzymic activity between pH 5 and 7·5 (Kimmel & Smith, 1954): 0·2 ml. aliquots of a minimal-medium killer solution and a purified-killer-factor solution were added to 0·2 ml. aliquots of an activated papain (crystalline B.D.H.) solution containing 0·025 M-cysteine and 0·005 M-versene; an inactive papain solution (papain alone); and a solution containing cysteine and versene. The solutions were incubated at 20° and the killer activity determined after 0, 4, 6 and 20 hr.

The results shown in Table 5 indicate that only the killer factor was inactivated by papain. Since it was inactivated by papain only in the presence of the reducing agents it is concluded that the proteolytic action of the enzyme was responsible. The eventual

loss of killer activity by the purified killer control sample was due to the unstable nature of the fractionated killer factor.

Mode of action of the killer factor. The susceptibility of the sensitive cells was studied by comparing the % killing at different stages in the growth cycle. The sensitive cells were most susceptible during active growth but were completely resistant during stationary phase (Woods, 1966). Similar effects associated with changes in growth rate are well known in bacteria (*Topley & Wilsons Principles* 1964).

Table 5. *Effect of the protease enzyme, papain, on the killer factor*

Sample	Relative concentrations of active killer (log killer concentration)		
	0 hr	6 hr	20 hr
Killer factor + MM* + gelatine	1·40	1·40	1·40
Killer factor + MM − gelatine + cysteine + versene	1·40	1·40	1·05
Killer factor + MM − gelatine + papain	1·40	1·40	1·40
Killer factor + MM + gelatine + cysteine + versene + papain	1·40	0·30	0
Purified killer factor	0·53	0·30	0
Purified killer factor + papain	0·53	0·30	0
Purified killer factor + cysteine + versene + papain	0·53	0	0

* Minimal medium.

Table 6. *Effect of temperature and pH on the post-absorption action of the killer factor*

Exp.	Type of medium	Post-absorption treatment		(%) Killing
		pH	Temp.°	
1	Sensitive	4·8	22	0
		5·8	38	0
	Killer	4·8	22	57·5
		5·8	22	43·0
		4·8	28	51·2
		5·8	28	31·0
		4·8	38	25·2
		5·8	38	18·7
2	Sensitive	4·8	18	0
		4·8	38	0
	Killer	4·8	18	78·0
		4·8	24	72·7
		4·8	28	68·5
		4·8	38	60·4

The action of the killer factor was investigated under different pH and temperature conditions. Sensitive cells were added to samples of killer and control solutions. The cells were plated on media buffered at pH 4·8 and/or 5·8 and incubated at 18°, 22° 24° 28° or 38°. The % of sensitive cells killed was estimated by comparing the number of colonies arising on killer and control plates.

The results (Table 6) show that the post-absorption action of the killer depended on the growth conditions, and was not necessarily fatal unless the organisms were exposed to the optium conditions for the stability of the killer factor. It is interesting to note that the effect of temperature on the post-absorption action of the killer factor, contrasts with that on the action of megacin where post-adsorption death was proportional to the incubation temperature (Holland, 1962).

Treated sensitive yeast cells kept at 0° for 0–72 hr on agar plates buffered at pH 4·8 did not subsequently recover when the cells were allowed to grow at 22° (Woods, 1966).

Preliminary experiments indicated that the action of the killer factor was not reversed by exposing treated cells to papain. Further, washing and shaking treated cells did not affect the post-absorption action of the killer factor. This contrasts with the action of colicins, which is reversed by trypsin, suggesting that they are adsorbed on the cell surface (Nomura, 1964).

DISCUSSION

The killer factor in yeast, *Saccharomyces cerevisiae*, may be compared with any other agents involving killing and heredity, but the analogy between the killer factor and bacteriocins is particularly striking. Both act on strains of the same or closely related species and are proteinaceous in nature. The production and stability of the killer factor and some bacteriocins is very dependent on growth conditions, which must be carefully controlled for optimal titres. The killer factor is markedly more stable in agar medium than in broth. Similarly, many colicinogenic strains which produce good zones of inhibition on agar may show little or no activity in broth (Reeves, 1965).

The proteinaceous nature of the purified killer factor is indicated by its inactivation by papain, its absorption at 260 and 280 mμ, and the Folin–Ciocalteu test (1927) for proteins described here. The presence of aromatic moieties in the killer factor is suggested by both the significant absorption observed at 280 mμ (Layne, 1957) and the adsorption of the factor by Sephadex. It is well known that Sephadex adsorbs predominantly aromatic and heterocyclic compounds (Gelotte, 1960).

The size of the killer factor is at present unknown but the dialysis experiments suggest that it is a macromolecular protein.

The bactericidal action of the killer factor and that of megacin are very similar in many respects. First, growth of the treated sensitive organisms is required before death occurs. Secondly, the physiological state of the sensitive cells affects the amount of killing, and, thirdly, absorption and death of the organisms are separated into two distinct processes. Holland (1962) reported that the decrease in sensitivity, with age, of *Bacillus megaterium* strains to megacin is concurrent with the progressive development of an extensive slime layer. He suggested that the slime layer prevented or retarded the penetration of megacin to those structures where it exerted its lethal activity. At present the reason for the increased sensitivity of the sensitive yeast cells to the action of the killer factor during log phase is not known.

The post-absorption action of both the killer factor and megacin is affected by the growth conditions. The former requires that the organisms be exposed to the optimum conditions for the stability of the killer factor during growth, but the latter is not necessarily fatal unless the organisms are exposed to optimum growth conditions. Holland (1962) suggested the possible involvement of an enzymic system since the post-absorption death rate is proportional to the incubation temperature, and the

$Q_{10}(27–37°)$ for the loss of viability is close to 2. The effect of temperature between 28° and 38° on the action of the killer factor has the opposite effect resulting in a decrease in the % killing.

Another example of the effect of the physiological state of the sensitive and temperature organisms on the activity of the bacteriocin, pesticin, produced by *Pasteurella pseudotuberculosis* was reported by Ben-Gurion & Hertman (1958). They indicated that the activity of pesticin is 20 times greater when tested at 37° than at 30°. However, it should be noted that the sensitive organism *P. pseudotuberculosis* grows more abundantly at 37° than at 30°.

The authors wish to thank Mr J. Kirkham, who carried out the electron microscopy, and the Science Research Council for financial support of this work. One of us (D. R. W.) wishes to acknowledge a Rhodes Scholarship.

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J. gen. Microbiol. (1968), **51**, 127–135
Printed in Great Britain

Chlorophyll Formation in *Euglena gracilis* var. *bacillaris*: Interference by Vitamin Analogues

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(*Accepted for publication* 22 *September* 1967)

SUMMARY

The formation of chlorophyll in non-proliferating etiolated cells of *Euglena gracilis* var. *bacillaris* is inhibited by some antimetabolite analogues of vitamins. The cell size and cell mass of light-grown euglenas is considerably increased in vitamin B 12 deficiency. The inhibitory effect of 2,6-diaminopurine on chlorophyll formation in non-proliferating euglenas was not annulled by vitamin B 12; 6-mercaptopurine, sulphanilamide and benzimidazole were without effect. Isoniazide inhibition is not reversed by niacin; surprisingly, the vitamin itself is markedly inhibitory. The inhibitory effect of niacin, however, is prevented by pyridine-3-sulphonate. Niacin and its analogues were more inhibitory to growth in the dark than in the light. The inhibition of growth by niacin in the light is annulled appreciably by either glucose or pyruvate, or an overwhelming concentration of vitamin B 12. Aminopterin, desoxypyridoxine and 2-chloro-*p*-aminobenzoic acid do not have any effect on chlorophyll synthesis in non-proliferating euglenas; the last mentioned inhibits the growth of the alga more in the dark than in the light. Thiamine deficiency inhibits growth; such sub-optimally grown euglenas also synthesize less chlorophyll per cell on subsequent illumination under non-proliferation conditions.

The negative growth response of light-grown *Euglena gracilis* var. *bacillaris* to niacin suggests a microbiological method of estimating this vitamin in biological materials and pharmaceutical preparations up to concentrations of 70 μg./ml. in the growth medium. The 50% inhibition level of niacin in the light is 46 μg./ml.

INTRODUCTION

Exposure of dark-grown euglenas to light in a non-proliferation medium brings about the synthesis of chlorophyll with several associated changes. The formation of chlorophyll is inhibited by antimetabolite analogues of purines, pyrimidines and amino acids (Dubash & Rege, 1967*a*). No direct evidence of the involvement of vitamins during the pigment formation in dark-grown non-proliferating euglenas is available; such an apparent independence may mean synthesis of these during growth irrespective of illumination. The effect of antimetabolite analogues of vitamins was therefore studied with a view to explore the possible association of vitamins with the synthesis of chlorophyll.

METHODS

Euglena gracilis var. *bacillaris* was grown, harvested and illuminated under non-proliferating conditions as described earlier (Dubash & Rege, 1967*a*, *b*). Measurement

of growth, chlorophyll and cell numbers and all other experimental details have already been described. Dry weights of euglenas were determined by drying samples of the washed cell suspensions at 50–60° for 12–14 hr.

RESULTS

Requirement of vitamin B 12 and the effect of its antimetabolite analogues on growth and chlorophyll synthesis

Euglena gracilis var. *bacillaris* exhibits a unique requirement of an exogenous supply of vitamin B 12 (Hutner & Provasoli, 1964); it was of interest to check whether this vitamin is required for chlorophyll synthesis or for other metabolic purposes.

Organisms grown under conditions varied with respect to vitamin B 12 showed variations in their chlorophyll content; sub-optimally grown organisms had a lower chlorophyll index per mg. dry wt. However, when the chlorophyll index was measured per organism, a slight enhancement was observed in the case of sub-optimally grown

Table 1. *Effect of vitamin B 12 deficiency on the synthesis of chlorophyll in Euglena gracilis var. bacillaris grown in the light*

Vitamin B 12 in medium ($\mu\mu$g./ml.)	Dry wt cell suspension (mg./ml.)	Cell count of suspension ($\times 10^6$/ml.)	O.D. at 660 mμ of 10 ml. of methanol extract		
			Per ml. of suspension	Per mg. cells	Per 10^6 cells
50 (Optimal)	4·36	4·42	0·298	0·068	0·067
5 (Sub-optimal)	3·23	1·25	0·096	0·029	0·077

Table 2. *Comparison of dry weights of Euglena gracilis var. bacillaris grown under different conditions of stress*

Stress condition	Dry weight of 10^6 cells (mg.)	$\frac{\text{Dry wt of treated cells}}{\text{Dry wt of control cells}} = N$
Control*	0·99	1
Vitamin B 12 deficiency*	2·58	2·61
Control†	1·54	1
Grown at 13·3–17 °C.†	3·78	2·46

* *Euglena gracilis* var. *bacillaris* grown in light in the glutamate-malate medium of Hutner, Provasoli, Schatz & Haskins (1950).

† Readings of Buetow (1962): *Euglena gracilis* var. *bacillaris* (SM-LI), a streptomycin bleached mutant, grown in the dark with acetate as the sole carbon source in the medium of Cramer & Myers (1952).

euglenas (Table 1). This indicated an increase in size and cellular mass in vitamin B 12 deficiency. Buetow (1962) observed a similar decrease in the growth rate and increase in cellular mass of euglenas at lower incubation temperatures. Table 2 shows a comparison of the growth characteristics of the euglenas grown under different conditions of stress.

Although vitamin B 12 deficiency distorts cell metabolism and brings about these characteristic biochemical lesions during growth, results in Table 3 show that the

vitamin plays no significant role during chlorophyll synthesis in non-proliferating organisms. The 2,6-diaminopurine inhibition of chlorophyll synthesis was not annulled to any extent by the presence of vitamin B 12. 6-Mercaptopurine, sulphanilamide and benzimidazole do not inhibit pigment synthesis.

Table 3. *Effect of anti-metabolite analogues of vitamin B 12 on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Chlorophyll synthesized/cell/72 hr (%)	
	Control	+ 1 mμg./ml. B12
None	100	100
2,6-Diaminopurine	63·9	65·0
6-Mercaptopurine	114·0	102·8
Sulphanilamide	102·8	95·2
Benzimidazole	97·2	80·5

All analogues were added at a concentration of 1×10^{-3} M in the non-proliferation medium.

Table 4. *Effect of antimetabolite analogues of vitamins on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Concentration in the non-proliferation medium (μg./ml.)	Inhibition of greening (%)
None	.	0
Isoniazide	2000	65·5
3-Acetylpyridine	200	−11·5
Pyridine-3-sulphonic acid	200	−17·1
Aminopterin	20	10·7
2-Chloro-*p*-amino benzoic acid	25	0
Desoxypyridoxine	100	4·9

Table 5. *Effect of anti-metabolite analogues of vitamins on the growth of Euglena gracilis var. bacillaris*

Analogue	Concentration in the growth medium (μg./ml.)	Inhibition of growth (%)	
		Light	Dark
None	.	0	0
Niacinamide	50	14·0	68·5
Isoniazide	50	−6·0	14·6
3-Acetylpyridine	50	44·5	76·0
Pyridine-3-sulphonic acid	50	0	22·4
Niacin	50	62·0	75·4
Aminopterin	20	3·1	1·5
2-Chloro-*p*-amino benzoic acid	25	54·0	63·8

Effect of antimetabolite analogues of vitamins on chlorophyll synthesis and growth of Euglena gracilis var. bacillaris

Several other antimetabolite analogues of vitamins were tested for their effect on chlorophyll synthesis (Table 4) and growth (Table 5) of *Euglena gracilis* var. *bacillaris*. Aminopterin and desoxypyridoxine had little effect on chlorophyll synthesis in non-

proliferating euglenas. Among the niacin derivatives tried, only isoniazide was inhibitory, whereas 3-acetylpyridine and pyridine-3-sulphonate were, in fact, stimulatory. However, these had varying effects on the growth of the euglenas. 2-Chloro-*p*-aminobenzoic acid and 3-acetyl pyridine were quite inhibitory to growth (Table 5) and more so in the dark, whereas pyridine-3-sulphonate and isoniazide were only inhibitory in the dark; aminopterin was ineffective. 2-Chloro-*p*-aminobenzoic acid and sulphanilamide had no effect on chlorophyll synthesis in non-proliferating euglenas although both inhibited growth.

Effect of niacin derivatives

In an attempt to study the possible annulment by the vitamin of the inhibition of chlorophyll synthesis by the antimetabolite analogues of niacin it was observed that 200 μg./ml. niacin was itself inhibitory to the synthesis of chlorophyll in non-proliferating euglenas. The effects of the various antimetabolite analogues of niacin along with this vitamin were therefore studied (Table 6). Figure 1 shows the negative growth-response relationship between niacin concentration and growth of *Euglena gracilis* var. *bacillaris*.

Table 6. *Effect of niacin and its anti-metabolite analogues on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Concentration in the non-proliferation medium (μg./ml.)	Inhibition of greening (%)
None	.	0
Niacin	200	95·6
Niacinamide	2000	100
Isoniazide	2000	65·6
3-Acetylpyridine	200	−11·5
Pyridine-3-sulphonic acid	200	−17·1
Niacin + 3-acetylpyridine	.	100
Niacin + pyridine-3-sulphonic acid	.	−54·1

Both niacin and its amide were inhibitory to growth as well as chlorophyll synthesis (Tables 5, 6). The growth inhibition with niacin was more marked in the dark than in the light as with other antimetabolite analogues of this vitamin. The inhibition of chlorophyll synthesis by niacin was completely overcome by pyridine-3-sulphonate (Table 6). Attempts to reverse this anomalous inhibition of growth by niacin have been met with only partial success. Table 7 shows a 50 % annulment of the inhibition in the light by glucose or by a hundredfold increase in vitamin B 12 in the growth medium. There was, however, no annulment by these supplements in the dark.

Among the several Krebs-cycle intermediates tested for their effect on the niacin inhibition of growth, only pyruvate annulled the inhibition completely in light and partially in the dark. β-Glycerolphosphate and α-ketoglutarate, however, could partially annul the niacin inhibition in the light but not in the dark. Succinate, citrate, malate and fumarate all aggravated the inhibition, particularly in the light. These results are given in Table 8.

Effect of thiamine

The effect of thiamine deficiency on growth and chlorophyll synthesis in *Euglena gracilis* var. *bacillaris* is shown in Table 9. Thiamine-deficient organisms had a 15% lower potential for chlorophyll synthesis than normal organisms when they were illuminated under non-proliferating conditions.

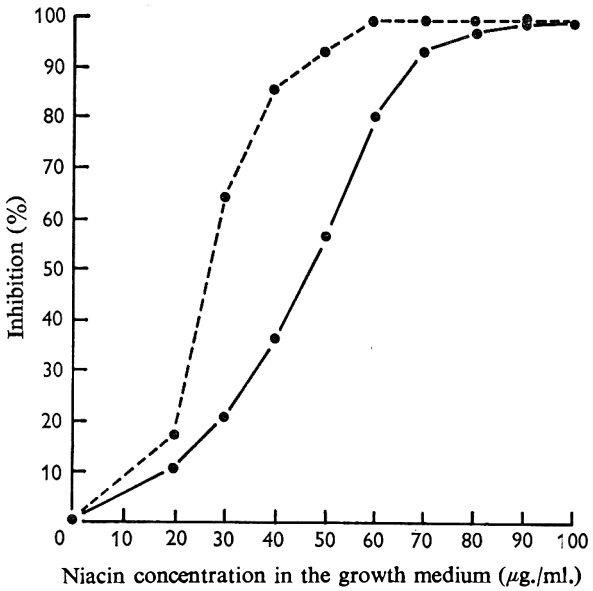

Fig. 1. Effect of niacin on the growth of *Euglena gracilis* var. *bacillaris* in the light (——) and in the dark (– – – –).

Table 7. *Annulment of niacin growth inhibition of Euglena gracilis var. bacillaris with glucose and vitamin B 12*

Vitamin B 12 in medium (μμg./ml.)	Niacin in medium (46 μg./ml.)	Glucose 1%	Growth in (Klett) Light	Growth in (Klett) Dark	Inhibition of growth (%) Light	Inhibition of growth (%) Dark
50 (normal)	–	–	179	.	0	.
	–	+	182	240	0	0
	+	–	90	.	50	.
	+	+	133	17	26	93
5000 (100-fold)	–	–	186	.	0	.
	+	–	135	.	27	.
	–	+	.	276	.	0
	+	+	.	14	.	98

Assay of niacin with Euglena gracilis var. baccillaris

The *Euglena gracilis* assay of vitamin B 12 (Hutner, Bach & Ross, 1956) owes its world-wide acceptance to its extreme sensitivity and its specificity for vitamin B 12 (Hutner, 1961; Hutner & Provasoli, 1964). The inhibitory effect of niacin on the photosynthetic growth of the euglenas was striking and offered an elegant method for

assaying this vitamin microbiologically, taking advantage of the typical negative-growth response curve which is shown in Fig. 1. A linearity of response was shown for niacin concentrations up to 70 μg./ml. in the basal medium with a 50% inhibition level of the vitamin corresponding to 46 μg./ml.

Table 8. *Effect of Krebs cycle intermediates on the niacin inhibition of growth of Euglena gracilis var. bacillaris*

Krebs' cycle acid added* (1%)	Inhibition of growth with 50 μg./ml. niacin (%)	
	Light	Dark
None	65·0	83·5
Glucose	33·0	96·8
Pyruvate	0	45·8
Lactate	59·0	87·0
Succinate	84·0	84·0
Malate	78·0	89·5
α-Ketoglutarate	43·5	92·6
β-Glycerophosphate	37·5	87·0
Fumarate	78·8	92·3
Citrate	82·2	83·1

* Additions of Krebs' intermediates were to the basal medium containing glutamate (1%) and malate (0·2%) but without glucose.

Table 9. *Thiamine requirement for growth and greening of Euglena gracilis var. bacillaris*

Thiamine in growth medium ($\mu\mu$g./ml.)	Inhibition of growth (%)		Inhibition of chlorophyll synthesis in non-proliferating cells (%)
	Light	Dark	
0	46·0	16·0	15·0
1000 (basal)	0	0	0

DISCUSSION

Although Venkataraman, Netrawali & Sreenivasan (1965) suggested on the basis of incorporation of labelled ^{14}C-formate that in DNA synthesis in *Euglena gracilis* var. *bacillaris* vitamin B 12 has a greater role in the reduction of ribotides to deoxyribotides than in the reduction of formate to thymine methyl and that the vitamin B 12-dependent conversion of glutamate to β-methyl aspartate also contributed to thymine synthesis, its exact function in this organism still remains obscure. The role of vitamin B 12 in chlorophyll synthesis in euglena has not been explored. The dry weight of euglenas grown under conditions of vitamin B 12 deficiency (Table 2) increased nearly 2½-fold as was the case of cultures grown at low temperatures (Buetow, 1962). Gigantism was also apparent from results given in Table 1 on the basis of the chlorophyll index of the euglenas grown under sub-optimal vitamin B 12 conditions. Both higher optimal temperatures and adequate vitamin B 12 favour cell multiplication with a constant fragmentation of cellular material which prevents cell gigantism due to 'stagnation'. Cell enlargement has also been observed in the *z* strain of *Euglena gracilis* (Epstein, Weiss, Causeley & Bush, 1962) grown under sub-optimal vitamin B 12 conditions. The occurrence of a wide variation in cell size in response to anti-metabolites or metabolite

deficiency has been reported by several workers (Beck, Hurlock & Levin, 1960; Dubash & Rege, 1967*b*; Epstein & Weiss, 1960*a*; Ford & Goulden, 1959; Rueckert & Mueller, 1960). Such varied size differentiation, along with the cellular accumulation of materials which render the organisms more opaque, must throw doubt on turbidimetric growth measurements. The vitamin B 12 assay with Euglena (Hutner *et al.* 1956) would, in the light of these experiments, be much more reliable if chlorophyll were taken as the index of growth rather than turbidity. The chlorophyll content of vitamin B 12-deficient organisms did not show such a wide variation (Table 1). The strong inhibition by acetate and niacin (see Coelho, Dubash & Rege, 1964) both of common occurrence in biological materials and pharmaceutical preparations and of growth stimulation by various Kreb's intermediates (Coelho *et al.* 1964) suggests need for caution when assaying vitamin B 12 or niacin with this alga. The graded negative growth response of photosynthetically growing *Euglena gracilis* var. *bacillaris* to the presence of niacin may prove useful for the assay of this vitamin in biological materials and pharmaceutical preparations.

Vitamin B 12, however, does not seem to have any significant role in chlorophyll synthesis in non-proliferating euglenas as is shown by the inability of vitamin B 12 to annul the inhibition by 2,6-diaminopurine. Although in growing euglenas, 6-mercaptopurine competes with vitamin B 12 presumably by interfering with dimethylbenzimidazol function rather than inducing purine deficiency (Epstein & Timmis, 1963; Epstein & Weiss, 1960*a*, *b*), no inhibition of chlorophyll synthesis by this antimetabolite analogue was observed (Table 3). Similarly, benzimidazole (Epstein & Timmis, 1963) and sulphanimide (Coelho, 1966), reported to have an anti-B12 activity for growth of Euglena, had no appreciable effect on greening. Epstein & Timmis (1963) have shown both aminopterin and amethopterin to be inactive for Euglena. Aminopterin and desoxypyridoxine have also no effect on chlorophyll synthesis in non-proliferating etiolated euglenas on illumination (Tables 4, 5).

Both niacin and its amide are inhibitory for growth as well as chlorophyll synthesis in Euglena (Tables 5, 6) and these as well as the other anti-metabolite analogues of vitamins are more inhibitory in the dark than in the light. This indicates that these inhibit some system of general metabolic significance and do not obstruct some step in chlorophyll biosynthesis. The inhibition of chlorophyll synthesis by niacin is completely annulled by pyridine-3-sulphonate. It is clear from Tables 5 and 6 that other substitutions in the β-position of the pyridine ring lower the toxicity of the compound. A probable site of action of niacin and niacinamide would be for the TPN-requiring glyceraldehyde-3-phosphate dehydrogenase formed during chlorophyll synthesis (Fuller & Gibbs, 1959; Brawerman & Konigsberg, 1960). However, the niacin inhibition is exerted even in the dark when the euglenas are grown heterotrophically. Schopfer & Keller (1951) have reversed the growth inhibition of *Euglena gracilis* caused by vitamin K 3 with niacin or niacinamide and Frank *et al.* (1963) have overcome the thalidomide growth inhibition in this alga by niacin, niacinamide, NAD and vitamin K 1. Although the latter authors have used a level of 10 μg./ml. niacin, they fail to report any inhibition of growth, whereas from Fig. 1 it is apparent that a 10% growth inhibition does occur at such a level. Frank *et al.* (1963), however, did find 6-amino-niacinamide to inhibit the growth of euglenas and reversed this effect with niacin. In the present case it is not only that a usual metabolite for other organisms is acting as an inhibitor, but that a normal antimetabolite ana-

logue, pyridine-3-sulphonate, annuls this inhibition. The reversal, though partial, of niacin toxicity to the photosynthetic growth of euglenas by overwhelming concentrations of vitamin B 12 may suggest that the former interferes in the utilization of the latter. It is also possible that vitamin B 12 is rendered unstable in the presence of niacin, particularly during sterilization of the medium at high temperatures; such examples of non-compatibility are known to occur in pharmaceutical preparations. The reversal by glucose of this niacin inhibition (Table 7) in the light may suggest that the inhibitor obstructs the photosynthetic pathway for eliciting energy; however, the more pronounced inhibition in the dark is inconsistent with this. The extremely high activity of pyruvate and α-ketoglutarate, particularly in the light to prevent niacin inhibition (Table 7), may suggest that niacin interferes in some manner with the pyridine nucleotide metabolism. Both these α-keto acids normally serve to produce the reduced NADH. It is quite possible that vitamin B 12 may act by achieving the same end-results, as it is known that this vitamin serves in maintaining sulphydryl groups in the reduced form (Dubnoff & Barton, 1956). A similar explanation had been given for the inhibitory effect of thalidomide (Frank *et al.* 1963), which is claimed to interfere at a point where niacin is converted to NAD or with the utilization of NAD and vitamin K in cellular oxidations. Mention must also be made at this point of another observation of Coelho (1966) that many glucose metabolites, particularly α-ketoglutarate, spare the requirement of vitamin B 12 in this alga.

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Printed in Great Britain

Competition and Synergism between Cholesterol and Cholestanol in Oospore Formation in *Phytophthora cactorum*

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(*Accepted for publication* 25 *September* 1967)

SUMMARY

Cholesterol promoted oospore formation in *Phytophthora cactorum*; very few oospores were formed when cholestanol was incorporated in the medium instead. Above a critical concentration of cholesterol, cholestanol competitively inhibited oospore formation by cholesterol when both were added to the medium together. At low cholesterol concentrations, the addition of some cholestanol increased the number of oospores; further addition of cholestanol was inhibitory. Cholesterol and cholestanol stimulated vegetative growth to a similar extent.

INTRODUCTION

The fungus *Phytophthora cactorum* (Leb. & Cohn) Schroet. grows vegetatively on an agar medium containing sucrose, asparagine, mineral salts and thiamine (basal medium); but for sexual reproduction and oospore formation sterols must be added to the medium. Different sterols vary greatly in their effectiveness in promoting reproduction. Some, classified by Elliott, Hendrie & Knights (1966) as 'active', promote oospore formation, but with others, called 'partially active', only oogonia and antheridia are formed: the contents of the oogonia degenerate and no oospores are formed.

The question arises as to whether a partially active substance added to the medium together with an active one suppresses or enhances the action of the active substance in promoting oospore formation. This paper reports the effect of mixtures of the active substance, cholesterol, and the partially active substance, cholestanol.

METHODS

The strain IMI 21168 of *Phytophthora cactorum* was used. The basal medium was that described by Elliott *et al.* (1966); it was solidified with 1% (w/v) Difco Bacto-agar. Cholesterol and cholestanol were dissolved in diethyl ether and added to the medium after it had been autoclaved.

The cholesterol and cholestanol were examined by gas–liquid chromatography (GLC) (see Knights, 1967, for details), and each appeared to be at least 99% pure. To test further for the presence of cholestanol in the cholesterol, the sample was oxidized with Jones's reagent (Bowden *et al.* 1946) so that the cholesterol was converted to Δ^4-cholesten-3,6-dione and cholestanol to cholestan-3-one. A trace of material was detected by GLC which might have been cholestan-3-one (Fig. 1), but again the

maximum possible amount was under 1 %. The presence of cholesterol in the cholestanol was tested for by the Liebermann–Burchard reaction. The intensity of colour developed indicated a maximum cholesterol content in the sample of 1·65 %. However, ultraviolet spectra of the colour reactions of the cholestanol sample and of pure cholesterol were not identical, allowance for dilution being made (Fig. 2). Cholesterol shows a peak at 415 mμ (absent from the cholestanol sample) and the spectrum changes with time (which did not occur with the cholestanol sample); moreover the cholestanol sample showed a weak peak at 455 mμ, absent from cholesterol. Thus cholesterol content of the cholestanol is likely to be well under 1 %.

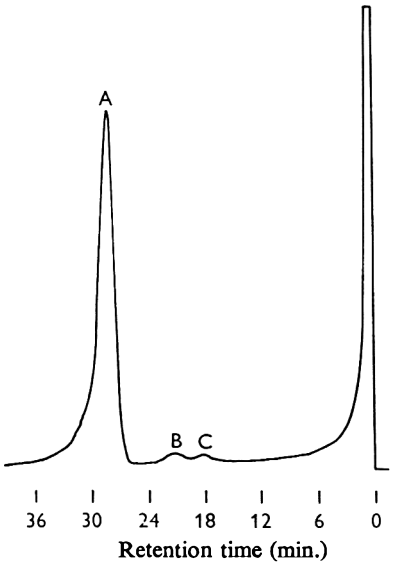

Fig. 1

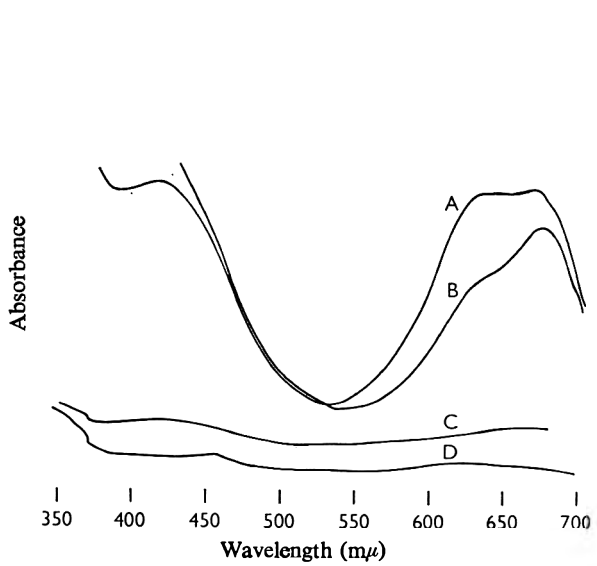

Fig. 2

Fig. 1. GLC analysis (on 1 % SE-30 at 250°) of oxidation product of cholesterol sample. Peak A, cholest-4-ene-3,6-dione; B, cholest-4-ene-3-one; C, cholestan-3-one ?+unreacted cholesterol.

Fig. 2. Spectra of Liebermann–Burchard reaction mixtures of A, cholesterol (1·6 mg./2 ml.); B, same after about 30 min.; C, cholesterol, diluted 1·6 in 100; D, cholestanol (6·87 mg./2 ml.).

The fungus was grown in small 'Monax' Petri dishes, 5 cm. diam. (nominal), containing 5 ml. medium. The inoculum was a disc 4 mm. diameter cut from a culture growing on basal medium. Incubation was at 24° in the dark.

Oospore counts were made in cultures 3 weeks old. By this time oogonia either contained oospores, or their contents had degenerated. For counting, the Petri dishes were inverted on the microscope stage and a convenient plane of focus was selected (generally just inside the glass). The Petri dish was traversed under the low-power objective from the outer edge of the colony to the centre, the fine-focus knob being continuously adjusted through one quarter of a turn only.

For statistical tests, the individual oospore counts, x, were transformed to $(x+\frac{1}{2})^{\frac{1}{2}}$. It is these transformed values which are plotted in Fig. 3 and 4.

RESULTS

In the first three experiments (Fig. 3) the concentration of cholestanol was arranged to be 0, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, ..., 64 times that of cholesterol (w/w), the amounts of cholesterol added being 10, 1 and 0·2 mg./l. in the different experiments. At cholesterol 10 mg./l., the addition of even a small proportion of cholestanol decreased the oospore count. At cholesterol 0·2 mg./l., the addition of some cholestanol promoted oospore formation; there was a maximum at a ratio 2:1 cholestanol:cholesterol, above which the oospore count decreased. At cholesterol 1 mg./l. no promoting effect of cholestanol was observed, but there was only a small decrease in the oospore count until the ratio cholestanol:cholesterol exceeded 2:1.

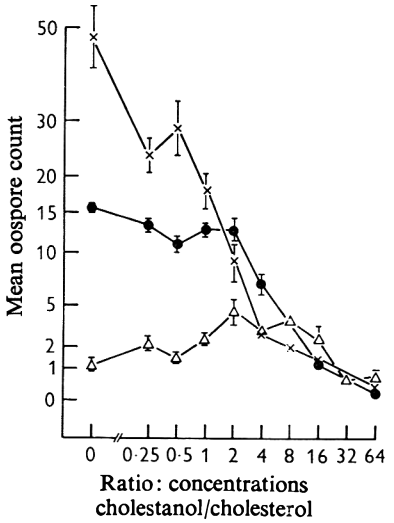

Fig. 3

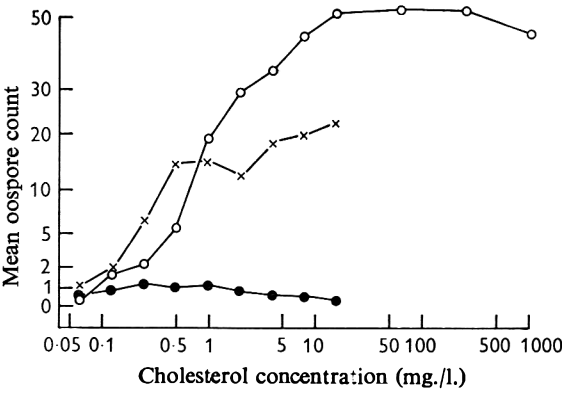

Fig. 4

Fig. 3. Effect on oospore numbers in *Phytophthora cactorum* of adding cholestanol to medium containing cholesterol: × —— ×, 10 mg./l. cholesterol; ●——●, 1·0 mg./l. cholesterol; △——△, 0·2 mg./l. cholesterol.

Fig. 4. Oospore numbers of *Phytophthora cactorum* in relation to concentration of cholesterol (○——○), cholesterol+cholestanol 1:2 (×——×) and cholestanol (●——●). The figures on the scale are to be doubled to give the cholestanol concentration appropriate to each point.

In the next experiment (Fig. 4) the ratio of the amounts of cholestanol and cholesterol was fixed at 2:1, but the actual amounts varied. Control cultures were grown with the same amounts of cholesterol and cholestanol separately, and in this experiment the effect of high concentrations of cholesterol on oospore formation was also tested. From Fig. 4 it can be seen that the number of oospores increased with increasing cholesterol concentration up to about 10 mg./l. Further increase in the amount brought about no significant change in the number of spores. At the lower concentrations (less than 2 mg./l.) the oospores were confined to the outermost part of the colony. At higher concentrations they occurred in increasing numbers in the central region. The curves for oospore number for cholesterol alone and for the cholesterol+cholestanol

mixture intersected at about cholesterol 0·9 mg./l. Above this concentration the addition of twice as much cholestanol decreased the number of oospores; below it, the addition of twice as much cholestanol increased it.

In these experiments a very few oospores were found in the cholestanol controls, contrary to our previous experience (Elliott *et al.* 1966). The use of Monax glassware rather than Pyrex as in our previous work was found not to be the explanation of the difference. The curve relating oospore number to cholestanol concentration (Fig. 4) did appear to have a maximum, but the numbers of spores did not vary significantly between concentrations, and were in all cases very small indeed. No oospores were seen in the controls without either sterol.

Table 1. *Phytophthora cactorum: mean oospore counts in media with different concentrations of cholesterol and cholestanol*

Each value is the mean of four counts in each of eight Petri dishes; untransformed data.

Concentration of cholestanol (mg./l.)	Concentration of cholesterol (mg./l.)				
	0·5	1	2	5	10
0	6·78	18·75	27·31	42·59	56·81
1	11·97	13·78	26·03	27·97	48·22
5	4·00	9·94	12·72	26·31	38·78

Analysis of variance (data transformed, $[x+1/2]^{\frac{1}{2}}$)

	D.F.	Mean square	*F*
Between cholesterol concentrations	4	230·99	49·56**
Between cholesterol concentrations	2	67·49	13·94**
Interaction 1	2	16·53	3·41*
Interaction 2	6	4·17	—
Between replicate Petri dishes	105	4·84	4·22**
Within dishes	360	1·15	—

Interaction 1 compares the effect of cholestanol concentration at 0·5 mg./l. cholesterol with its average effect at the other cholesterol concentrations. Interaction 2 compares the responses to cholestanol at the four higher cholesterol concentrations.

* *P* 0·05–0·01. ** *P* very small.

The methods of enzyme kinetics have been applied to the growth of higher plants in response to auxins (Foster, McRae & Bonner, 1952; McRae, Foster & Bonner, 1953). These authors substituted the rate of elongation for the rate of the reaction in the enzyme system, and the concentrations of auxin and anti-auxin for the concentrations of enzyme substrate and inhibitor. It seemed appropriate to treat the present case similarly, using the oospore count in place of the rate of reaction (v) and concentrations of cholesterol and cholestanol for the concentration of substrate (s) and inhibitor (i), respectively, although the oospore count itself is not strictly a rate.

The data of Table 1 have been plotted in two ways which are convenient for distinguishing competitive from non-competitive inhibition (Dixon & Webb, 1964), namely $1/v$ against $1/s$ (Fig. 5) and s/v against s (Fig. 6). The untransformed means were used for the v values. In Fig. 5 the points at which the lines for cholestanol concentrations 0 and 5 mg./l. cut the vertical axis do not differ significantly. In Fig. 6 the

lines for these two concentrations do not differ significantly in slope. These are the results expected in the case of competitive inhibition. For cholestanol 1 mg./l., the line would appear to lie between the lines for the other two cholestanol concentrations (i.e. 0 and 5 mg./l.), but to cross the no-cholestanol line at low cholesterol concentrations where the effect of adding cholestanol to the cholesterol is to increase the oospore count.

Vegetative growth (dry weight of mycelium) in media with cholesterol or cholestanol is compared in Table 2. The results were similar, but cholestanol appeared to be slightly less effective in promoting growth at low concentrations.

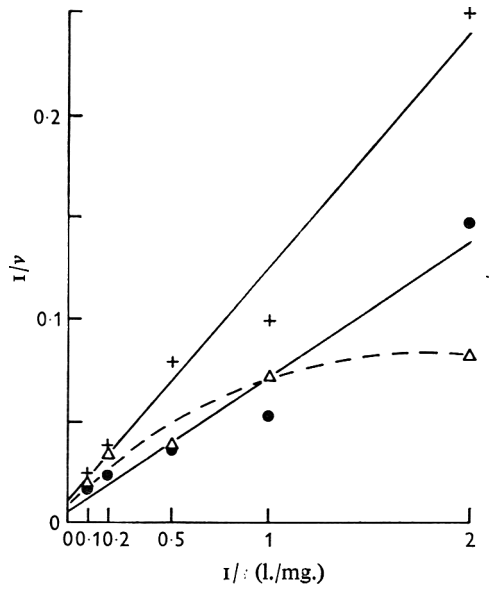

Fig. 5

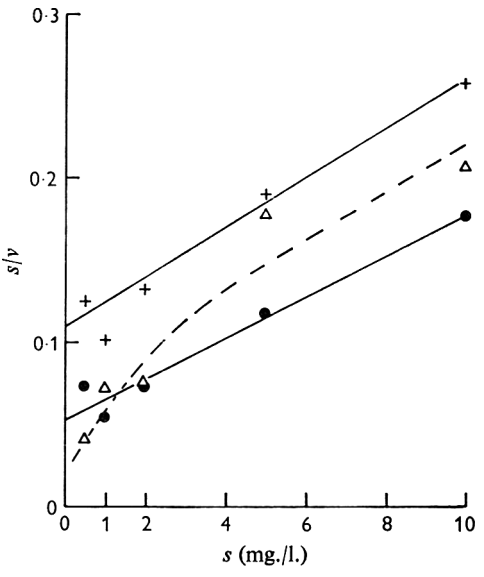

Fig. 6

Fig. 5. Relationship between reciprocal of mean oospore count ($1/v$) and reciprocal of cholesterol concentration ($1/s$): +——+, 5 mg./l. cholestanol (calculated line); △- - -△, 1 mg./l. cholestanol (line drawn by eye); ●——●, no cholestanol (calculated line).

Fig. 6. Relationship between ratio of cholesterol concentrations (s) to mean oospore count (v) and cholesterol concentration: symbols as in Fig. 3.

Table 2. *Effects of cholesterol and cholestanol on vegetative growth of Phytophthora cactorum*

Values given are dry weights (mg.) of mycelium harvested after incubation for 16 days (Exp. 1) or 13 days (Exp. 2) at 24° in 100 ml. flasks containing 25 ml. liquid medium.

Experiment		Concentration of sterol (mg./l.)			
		0	2·5	10	40
1	Cholesterol	44±4·0	97±6·7	118±5·7	110±4·1
	Cholestanol		73±4·9	92±7·5	111±3·9
2	Cholesterol	50±8·5	74±5·2	92±6·1	92±5·9
	Cholestanol		75±4·5	79±7·7	95±4·9

DISCUSSION

The results described above for the interaction of cholesterol and cholestanol in oospore formation in *Phytophthora cactorum* have many points of resemblance with the interactions between auxins and anti-auxins in higher plants. The results are, essentially, that at high concentrations of cholesterol the addition of cholestanol decreases the number of oospores; at low concentrations of cholesterol the addition of some cholestanol increases the oospore number, but larger amounts of cholestanol decrease the number again. For the interaction of indolylacetic acid (IAA) and phenylbutyric acid (PBA) in oat coleoptile segments, Skoog, Schneider & Malan (1942) found that at the lowest IAA concentration tested (0·005 mg./l.) the addition of some PBA resulted in an increase in length greater than that obtained with IAA alone, but adding more PBA was inhibitory. At higher IAA concentrations (0·05 mg./l. and above) the growth with added PBA was always less than with IAA alone. Thimann & Bonner (1948) found that high concentrations of tri-iodobenzoic acid inhibited, but low concentrations stimulated, growth induced by IAA. McRae *et al.* (1953) found that above a certain concentration of IAA the effect of adding 2,4-dichlorophenoxyacetic acid (2,4-D) was inhibitory to elongation of coleoptile segments; below that concentration of IAA, adding 2,4-D was stimulatory.

By analogy with the model proposed by McRae *et al.* (1953), it may be supposed that there are a number of sites in the cellular structure of Phytophthora which can be occupied by cholesterol or cholestanol, and at which these sterols perform their function in controlling oospore development. Above a certain critical concentration of cholesterol the addition of cholestanol displaces cholesterol from these functional sites and the oospore number is decreased. Below the critical cholesterol concentration the addition of cholestanol first results in more sites being occupied. (For this to lead to more oospores being formed, cholestanol must have some real, though low, activity in promoting the reactions concerned.) Addition of more cholestanol, however, again results in the displacement of cholesterol from the functional sites and its replacement with the much less active cholestanol, with concomitant decrease in spore number.

The promotion of oospore development is not the only effect sterols have on Phytophthora; indeed the results of adding sterols to Phytophthora closely resemble those found on adding a vitamin to a fungus which has a partial deficiency for it. It would appear, however, that Phytophthora cannot synthesize any sterol at all (Elliott, Hendrie, Knights & Parker, 1964; Hendrix, 1966). In Phytophthora sterols are required in the medium for normal development of sporangia and zoospores (Hendrix, 1965; Chee & Turner, 1965), and they also increase vegetative growth. The results reported in the present paper are consistent with the idea that there is only one kind of site occupiable by sterols and affecting oospore development, and different but related molecules can compete for these sites. However, it is possible that there is more than one kind of site which sterols can occupy. Cholestanol promotes vegetative growth only slightly less efficiently than cholesterol (Table 2; Elliott *et al.* 1966, Table 2), and both promote oogonium development, but they differ in their ability to promote the later oospore stages of the sexual process. The different specificities in molecular structure required for activity at these different stages suggest that sterols might exert their effect on them from different kinds of sites. The sterol requirement of insects can be met by a small amount of an essential sterol (cholesterol or 22-dehydrocholesterol) and a much

larger amount of a sparing sterol (cholestanol and many other substances; Clark & Bloch, 1959; Clayton & Bloch, 1963). The essential and sparing sterols would appear to have different functions, and they occur in the tissues in states differing in the degree of esterification and in the ease with which they turn over with excess sterol in the diet (Lasser, Edwards & Clayton, 1966). This clearly suggests that in insects sterols occupy more than one kind of site, a similar situation to that suggested for Phytophthora.

If in fact there are at least two kinds of sites, the increase in oospore numbers effected by cholestanol at low cholesterol concentrations is more readily explained than if there were only one kind of site. It may also be that any one kind of site has a higher affinity for more active molecules than for less active ones, so that the sites controlling oospore development will be occupied by cholesterol rather than cholestanol, except when cholestanol is in great excess. Further, during growth of the fungus the oospore sites may be synthesized or become functional after those which control oogonium formation. Thus with cholesterol alone and at low concentration many oospore sites will be unoccupied, but with addition of cholestanol, cholesterol will be displaced from the sites concerned with vegetative growth and oogonium development, and the number of oospore sites occupied by cholesterol increased.

I am indebted to Dr B. A. Knights for analysis of the samples of cholesterol and cholestanol.

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J. gen. Microbiol. (1968), **51**, 145–150
With 1 *plate*
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The Chemical Composition of the Cell Walls of *Penicillium digitatum* Sacc. and *Penicillium italicum* Whem.

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(*Accepted for publication* 27 *September* 1967)

SUMMARY

Purified cell walls were isolated from hyphae of *Penicillium digitatum* Sacc. and *P. italicum* Whem. Electron micrographs showed microfibrils in the walls. The walls are composed chiefly of carbohydrate material, mainly D-glucose, together with D-galactose, glucosamine and mannose. Only small amounts of protein were found. Fractionation with alkali and acid indicated that the walls contain several glycans.

INTRODUCTION

The composition, structure and biosynthesis of fungal cell walls have been investigated only to a limited extent (Aronson, 1965). It is known that the chemical and physical structure of these walls is complex, and that they are composed mainly of carbohydrate material, in the form of polymers of glucose, galactose, mannose and glucosamine (the latter as chitin) (Aronson, 1965; Sharon, 1965), but the various polymeric components have been isolated from the walls and characterized in a few cases only. The walls of two species of the genus *Penicillium* have been studied. Crook & Johnston (1962) analysed qualitatively the wall of *Penicillium notatum* and found glucose to be the major component; small amounts of galactose, glucosamine and mannose were also found. With walls of *P. chrysogenum*, Hamilton & Knight (1962) found mannose, galactose, glucosamine, glucose, xylose and rhamnose in the approximate molar ratios of 1:3:4·5:9:0·5:0·5. Fractionation with alkali and acid indicated that these walls had at least two chemically distinct polysaccharide layers.

Penicillium digitatum and *P. italicum* are the main pathogenic fungi which cause serious spoilage to stored citrus fruit in Israel, and improved methods for the control of these fungi are required. Since several antibacterial substances, notably penicillin, kill bacteria by specifically inhibiting cell-wall biosynthesis (Martin, 1966), it might be possible to develop antifungal agents which act in a similar way. For this purpose, a detailed knowledge of the chemical structure and biosynthesis of fungal walls may be necessary; as a first step we have examined the chemical composition of the walls of *P. digitatum* and *P. italicum*.

METHODS

Culture conditions. Spores of *Penicillium digitatum* Sacc. and of *P. italicum* Whem. were collected from 10-day single-spore cultures on potato glucose agar incubated at 25°. Spore suspensions in distilled water (10^6 spores/ml.) were used as inocula. Fern-

bach bottles containing 250 ml. potato glucose broth were inoculated with 2 ml. of spore suspension. After 20 hr of incubation at 25° the hyphae were harvested by filtration through cheesecloth and washed four times with cold distilled water. To get sufficient quantities of hyphae, 30 bottles were inoculated with *P. digitatum* spores and 6 bottles with those of *P. italicum*. The same culture media and techniques were used for growth of *P. chrysogenum*.

Preparation of cell walls. In a typical experiment, 1·2 g. of vacuum-dried *Penicillium digitatum* hyphae, 15 g. glass beads (Minnesota Mining and Manuf. Co., Type 130-5005) and 50 ml. water were stirred at top speed in a Servall Omnimixer cooled in an ice-water bath (Hamilton & Knight, 1962; Sharon & Jeanloz, 1964) for 1 hr. Disintegration was complete since no whole cells could be observed in samples examined under the microscope (× 1200). The mixture was then centrifuged in a Clay Adams Safe-guard centrifuge at low speed (80 *g*) for 5 min. and the precipitate (glass beads and intact cells) discarded. The supernatant fluid was centrifuged in the same centrifuge at high speed (800 *g*) for 20 min. The precipitate was suspended in water (24 ml.) and washed by differential centrifugation, first at low speed and then at high speed. Washing was repeated ten times; in the last few washes, no precipitate formed at low-speed centrifugation, and the supernatant fluid did not absorb at 260 mμ. The washed cell walls were then freeze-dried, yielding 213 mg. of a slightly grey fluffy material (18 % of the dry weight of hyphae used). *P. italicum* gave a similar yield.

Before freeze-drying, the walls were examined with the electron microscope. Electron micrographs were taken with an RCA EMU-2A electron microscope. Specimens were prepared on Formvar and carbon-coated grids. Shadow casting was with Pt at an angle of 1:4.

Fractionation of cell-wall components. The procedure used was based on the work of Hamilton & Knight (1962). Lipids were removed from the cell walls (200 mg.) by extraction with ether (50 ml.) for 2 hr in a Soxhlet extractor. The ether-extracted walls were dried in air at room temperature, suspended in N-NaOH (10 ml.) and the suspension stirred for 3 hr at room temperature. The walls were removed by centrifugation, and extracted again with alkali as before. The alkali extracts were pooled, ethanol (96 %, v/v, in water, 40 ml.) was added, and the mixture allowed to stand overnight at 2–4°. The precipitate which formed was collected by centrifugation, washed with ethanol (96 %, v/v, in water) in the centrifuge until neutral, and dried in vacuum over KOH and $CaCl_2$.

The residue which remained after alkali extraction was washed with water in the centrifuge until neutral, dried in vacuum, suspended in H_2SO_4 (72 %, v/v, conc. H_2SO_4 in water, 1 ml.) and the suspension allowed to stand at room temperature for 48 hr. The insoluble residue was collected by centrifugation and washed with water till neutral; the first water wash (12 ml.) was added to the 72 % H_2SO_4 extract. The combined extract was diluted to give a final concentration of N-H_2SO_4, and heated at 100° or 6 hr. The hydrolysis mixture was neutralized with solid $BaCO_3$, the precipitate removed by filtration and washed with a small volume of boiling water. The combined filtrates were freeze-dried.

For analysis of sugars, the ether-extracted walls and the various cell fractions were also hydrolysed as above (N-H_2SO_4, 6 hr, 100°) and neutralized in the same way.

Nitrogen was estimated by the procedure of Dumas (Steyermark, 1961) and *phosphorus* by the method of King (1932). *Ash* was estimated in samples which were oxi-

dized with fuming HNO_3 and then heated at 600° to constant weight. *Neutral sugar* was determined by the phenol method of Dubois *et al.* (1956), and *reducing sugar* by the method of Park & Johnson (1949), with glucose as standard.

Acid hydrolysates were analysed qualitatively by descending *paper chromatography* on Whatman No. 1 paper with the following solvent systems: (*a*) *n*-butanol + acetic acid + water (25 + 6 + 25 by vol.; upper phase); (*b*) isoamyl alcohol + pyridine + water (3 + 3 + 0·9 by vol.); (*c*) *n*-butanol + ethanol + water (4 + 1 + 1 by vol.); (*d*) *n*-butanol + pyridine + water (6 + 4 + 3 by vol.). Chromatograms were run for 18 hr. *Reducing compounds* were detected by the silver nitrate reagent (Sharon & Jeanloz, 1960) and amino sugars and amino acids by ninhydrin (0·5% in acetone). The nature of the various sugars present was inferred from experiments with appropriate authentic compounds as markers, and in the case of glucose and galactose this was substantiated by enzymic experiments (see below).

Glucosamine was estimated by the colorimetric Elson–Morgan method as modified by Gardell (1953) with D-glucosamine hydrochloride (Pfanstiehl) as standard. In some of the experiments, the glucosamine was separated from the neutral sugars with the aid of Dowex 50 (Boas, 1953). It was further identified by ion-exchange chromatography on the Beckman–Spinco amino acid analyser (Spackman, Stein & Moore, 1958). D-*Glucose* and D-*galactose* were estimated enzymically by the glucose oxidase (Glucostat) and galactose oxidase (Galactostat) reagents (Worthington Biochemical Corporation, Freehold, New Jersey), respectively. *Amino acids* were determined on the Beckman–Spinco amino acid analyser after samples had been hydrolysed for 20 hr in 6 N-HCl at 100° in sealed ampoules (Spackman *et al.* 1958).

RESULTS

The electron micrographs of the walls (Pl. 1, fig. 1, 2) indicate that they are rod-shaped, in accord with the typical morphology of hyphae of *Penicillium digitatum* and *P. italicum*. They exhibit a micro-fibrillar structure (cf. Aronson, 1965) which can clearly be seen in the internal part of the wall (Pl. 1, fig. 2). No distinct fibrillar structure was seen on the external surface of the walls. The gross structure of the wall and the fibrils was not affected by prolonged treatment with alkali (N-NaOH, 6 hr, room temperature).

The walls of both organisms were composed mainly of carbohydrates. Glucose, galactose, mannose and glucosamine were identified by paper chromatography of acid hydrolysates of cell walls; trace amounts of rhamnose and xylose were also present. Quantitative analysis showed (Table 1) that the major sugar was D-glucose, which comprised about one half of the weight of the walls. The other neutral sugars, D-galactose and mannose, accounted together for 12–14% of the weight. The proportions of these sugars, as well as those of glucosamine, did not differ markedly in the two organisms analysed. The main difference was in the quantity of ash. Whereas no ash was found in the walls of *Penicillium italicum*, the ash content of the walls of *P. digitatum* was consistently very high. A small part of this ash was accounted for by the presence of phosphate, but we have no explanation for the source of the bulk of the ash. It was not an artifact of the experimental procedure used, since no ash was found in the walls of *P. italicum*. Furthermore, we did not find any ash in walls of *P. chrysogenum* which we prepared.

The protein content of the walls appeared to be rather low, as could be inferred from the percentage of non-amino sugar nitrogen (total N minus amino sugar N) in the walls. This figure was 0·82 % for *Penicillium digitatum* and 0·20 % for *P. italicum*; using the factor 6·25, this corresponds to 5·1 % and 1·25 % protein in the walls of the two organisms, respectively. Analysis of acid hydrolysates (20 hr, 6N-HCl, 100°) of the walls on the amino acid analyser showed the presence of the typical protein amino acids, which amounted to 3 % of the weight of the walls of *P. digitatum* and to 1·5 % of those of *P. italicum*. A comparison of our results with those of Hamilton & Knight (1962) for walls of *P. chrysogenum* shows that, qualitatively, the composition of the walls of the three Penicillium species is similar. There were, however, some quantitative differences, notably in glucose, which was lower in *P. chrysogenum*, and in the content of galactose and glucosamine, which was significantly higher (up to 3 times) in the walls of this organism than in those of *P. digitatum* and *P. italicum*.

Table 1. *Analyses of freeze-dried cell walls of Penicillium digitatum and P. italicum after lipid extraction*

Total reducing sugar, D-glucose, D-galactose and glucosamine were estimated in the walls after hydrolysis (N-H_2SO_4, 6 hr, 100°) and neutralization of the hydrolysate with $BaCO_3$.

	P. digitatum	*P. italicum*
	% (w/v) of walls	
Reducing sugar (as glucose)	65·4	72·5
D-Glucose	45·4	51·6
D-Galactose	3·8	3·8
Glucosamine	5·7	9·0
Total nitrogen	1·27	0·90
Amino sugar N (calculated)	0·45	0·70
Phosphorus	1·8	0·13
Ash	29·5	< 0·5

Table 2. *Fractionation of cell-wall material from Penicillium digitatum and P. italicum*

	Wall fraction (%)	% in fraction			
		Neutral sugar	Glucose	Galactose	Glucosamine
			P. digitatum (130 mg.)		
Alkali-soluble	22·5	88	61	7	1
Acid-soluble	36	98	60	6·1	7·5
Residue	27	1	—	—	—
Total recovery	86				
			P. italicum (69·3 mg.)		
Alkali-soluble	30·3	90	65	4	0
Acid-soluble	53	71	58	5	16·5
Residue	2·9	18	4	—	—
Total recovery	86				

Extraction of the cell walls of *Penicillium digitatum* and of *P. italicum* with ether and evaporation of the solvent from the extract gave a small amount of solid residue (about 4 % of the weight of the walls), indicating a low lipid content. The cell walls were also fractioned with alkali and acid. Table 2 shows that alkali (N, two treatments of 3 hr each at 23°) extracted a fraction which comprised 22 % of the weight of

P. digitatum walls and 30 % of the walls of *P. italicum*. This fraction, composed mainly of glucose, might be a mixture of homo- or hetero-polymers.

Treatment with alkali did not affect the gross structure of the walls as seen with the electron microscope. This may indicate that the soluble polysaccharide(s) are not essential components of the rigid structure of the wall. When the fraction of the wall which was insoluble in alkali was put in 72 % (v/v) conc. H_2SO_4 in water for 2 days another portion went into solution—36 % for *Penicillium digitatum* and 52 % for *P. italicum*. A similar figure was found by Hamilton & Knight (1962) for walls of *P. chrysogenum*. Quantitative analysis of acid hydrolysates of this fraction revealed that it was composed mainly of glucose, with small amounts of galactose. It also contained glucosamine; it is possible that part of this glucosamine was in the form of chitin, since chitin is known to be soluble in concentrated sulphuric acid. This is in agreement with previous findings that Penicillium belongs to the group of fungi which contain chitin in their walls (Aronson, 1965). The part of the wall which was insoluble in 72 % (v/v) conc. H_2SO_4 in water did not contain any appreciable amount of carbohydrate; its amount in the case of *P. digitatum* was 27 % of the walls, and this fraction may have contained the bulk of the material corresponding to the ash. Only a small amount of acid-insoluble residue was found in the walls of *P. italicum* in accord with the absence of ash from these walls.

We wish to thank the members of the Section of Biological Ultrastructure for the electron micrographs.

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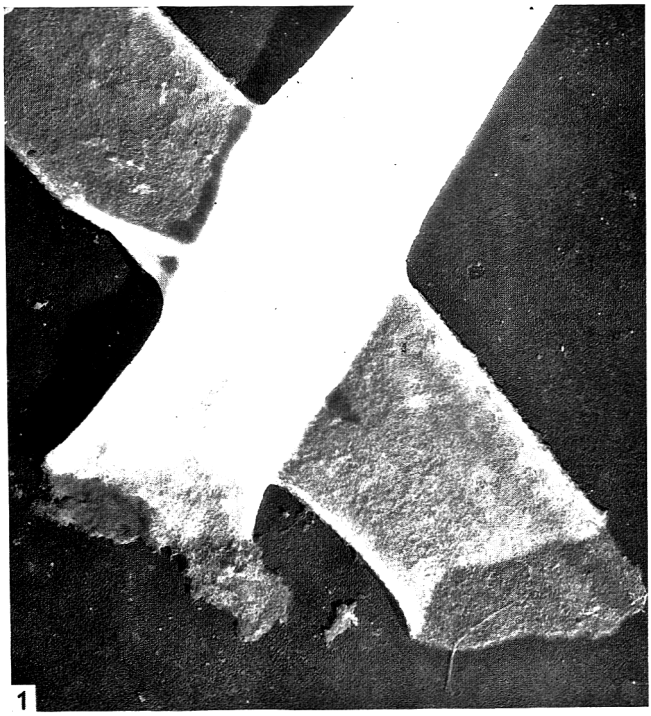
EXPLANATION OF PLATE

Electron micrographs of cell walls of *Penicillium digitatum* hyphae.

Fig. 1, magnification, ×5000. Fig. 2, magnification ×20,000: note the fibrous structure of the internal part of the cell wall.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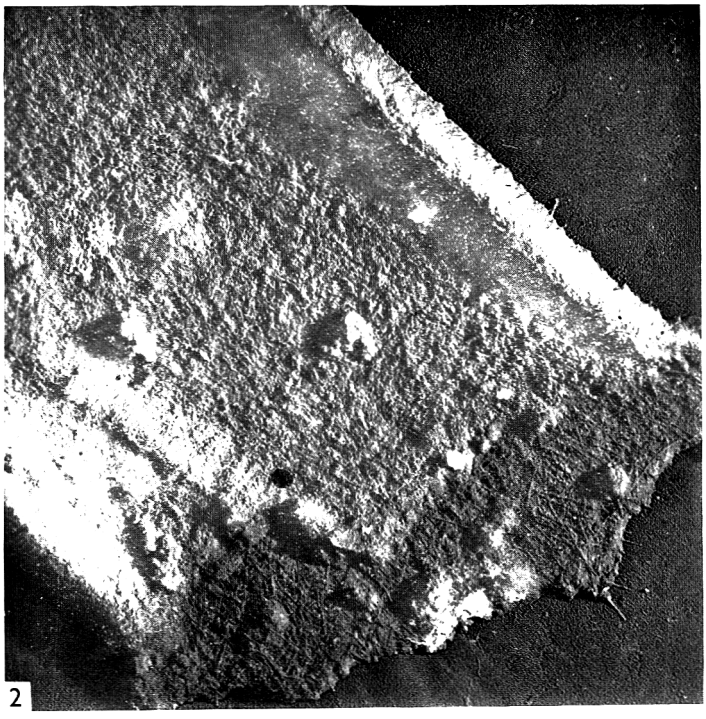
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J. gen. Microbiol. (1968), **51**, 151–158
Printed in Great Britain

Delineation of *Nocardia farcinica* by Delayed Type Skin Reactions on Guinea Pigs

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(*Accepted for publication* 28 *September* 1967)

SUMMARY

An immunological method based on the specificity of delayed-type skin reactions on guinea-pigs has been used for comparing 17 Nocardia strains. By this method, 4 isolates labelled *Nocardia farcinica*, four labelled *N. asteroides* and 1 strain labelled *N. blackwellii* could be grouped together with the type strain of *N. farcinica*. Three other strains labelled *N. farcinica* were clearly distinct from this group of 10 strains. A strain of *N. asteroides* used previously as reference strain for this species was clearly distinct from the *N. farcinica* strains, and this applied also to a recently proposed reference strain of *N. asteroides*. A strain of *N. brasiliensis* was clearly distinct from all the other strains, while the results obtained with a strain received without species designation were inconclusive. On this basis, the authors consider that all the 10 strains, which form a homogeneous group, should be classified as *N. farcinica*. The 3 other strains labelled *N. farcinica* should be classified into 2 other species. *Nocardia asteroides* would seem to be a separate species.

INTRODUCTION

In 1888 Nocard reported the isolation of a micro-organism which caused farcy in cattle in Guadeloupe (Nocard, 1888). The following year Trevisan described this organism under the name *Nocardia farcinica* (Trevisan, 1889). Based on a study of a culture (laboratory no. 752; see Table 1) believed to be Nocard's original and a similar strain (753) isolated and identified by Dr C. P. Fitch (New York) Waksman (1957) gave a more recent description of this species. Strain 752 is the type strain of *N. farcinica* (Sneath & Skerman, 1966).

Eppinger in 1891 described a new pathogenic micro-organism *Cladothrix asteroides* which was isolated from a man and which caused 'pseudotuberculosis' (Eppinger, 1891): this organism was named *Nocardia asteroides* by Blanchard (1896). A more recent description of *N. asteroides* is given by Waksman (1957). As an *ad hoc* reference strain of *N. asteroides*, strain 809 has recently been proposed (Sneath & Skerman, 1966).

Ørskov (1923) found differences between *Nocardia farcinica* (one isolate) and *N. asteroides* (three isolates) in morphology, and Erikson (1935), studying the same strain of *N. farcinica* as did Ørskov but two other strains of *N. asteroides*, later confirmed those observations. According to Waksman (1957), *N. farcinica* and *N. asteroides* can be distinguished by the colour of the vegetative mycelium. Mariat (1963) found some differences between *N. farcinica* and *N. asteroides* as regards capacity for synthesizing urease. On the other hand, in 1962 Gordon & Mihm published an expanded des-

cription of *N. asteroides* based on 142 strains, in which the 2 strains of *N. farcinica* mentioned above (752 and 753) could not be distinguished from those of *N. asteroides* by any of the observations or tests used.

Silvestri, Turri, Hill & Gilardi (1962) also referred *Nocardia farcinica* 752 to the same cluster ('sphere XI') as some strains of *N. asteroides*, including strain 809 mentioned above. However, this sphere XI also contained strains of several other species of *Nocardia* and one strain of *Mycobacterium phlei* (NCTC 8151). It is therefore probable that this sphere does not represent a single species in the sense in which this term has been used previously by other authors but is rather a taxon at a higher level. Thus it is not quite clear from previous studies whether *N. farcinica* and *N. asteroides* are, in fact, one or two species.

An immunological method, based on the specificity of the delayed type of skin reaction in guinea pigs, has been useful for classification of mycobacteria, and particularly for identification of species within this genus (Magnusson, 1961, 1965, Magnusson, Engbæk & Bentzon, 1961). By further application of this method on eight strains, it was found that distinction between *Nocardia asteroides* and *N. brasiliensis* was possible (Magnusson, 1962). The purpose of the present work was to see whether strains classified by other laboratories as *N. farcinica* and *N. asteroides* can be distinguished in the same way.

METHODS

Organisms. Strains classified as *Nocardia farcinica* or *N. asteroides* in various bacteriological laboratories or hospitals were included in the study, together with a strain of *N. brasiliensis* for comparison purposes. The origin and source of all the organisms used are shown in Table 1. Strain 752 is the type strain of *N. farcinica*, authentic co-type (Sneath & Skerman, 1966).

The cultures were maintained on Sauton medium in 250 ml. flasks at 38° and subcultured monthly.

Condition for growth and harvest. Cultures were inoculated by loop into 180 ml. Sauton medium in a 250 ml. flask and incubated at 38°. The cultures were incubated for 3–10 weeks, depending on the growth rate, and sterilized by heating in flowing steam for 1 hr. The organisms were harvested by paper filtration.

Immunological method. The experimental procedure was the same as that described previously (Magnusson, 1961; Magnusson *et al.* 1961), except for an amended method of sensitizing the guinea pigs and, in some cases culture filtrates instead of purified sensitins were used for the skin tests.

Sensitization of guinea pigs. One hundred and forty-eight albino guinea pigs (350–600 g.) were sensitized by intradermal injection of dried heat-killed organisms in liquid paraffin (Bayol 55). Each animal was given four simultaneous injections, each of 0·1 ml., of a suspension containing 0·4 mg. dried bacteria/ml.

Skin tests. Intradermal tests were done 3–4 weeks after sensitization, by using dilutions of filtrates of heat-sterilized cultures (cf) or purified sensitins (RS) prepared by a procedure similar to that used for tuberculin purified protein derivative. The culture filtrates were diluted 1/50 and 1/500 with isotonic phosphate-buffered saline (pH 7·38) containing 0·01 % chinosol and 0·005 % Tween 80 (Magnusson *et al.* 1958). Purified sensitins were used in doses of 2 μg. and 0·2 μg./0·1 ml. of the same diluent. Each injection consisted of 0·1 ml.

Table 1. *Strains studied*

Laboratory no.	Name when received, source* and culture collection numbers
80	*Nocardia* sp. SSC 261. Originally isolated from a Danish patient by Dr P. Holm, Statens Seruminstitut, Copenhagen. PH 184/45–46
81	*Nocardia asteroides* SSC 262. Originally isolated from a chicken by Dr O. J. Mayfield, D.V.M., Charles City, Iowa, U.S.A. EM 9935. McCL 93. RG 404
84	*N. asteroides* PH 598/51–52. Originally isolated from placenta of a cow, see Case II in Fey *et al.* (1954)
85	*N. asteroides* PH 599/51–52. Originally isolated from a dog, see Case I in Fey *et al.* (1954)
117	*N. brasiliensis* RG 774A. Originally isolated from a submandibular abscess (Gordon & Mihm, 1959). McCL 254
650	*N. asteroides* RG 784. Received at the NCTC, London, in June 1944 from N. F. Conant, Duke University Medical Center, Durham, North Carolina, U.S.A. NCTC 6761
677	*N. farcinica* G 396. IP 396
678	*N. farcinica* G 397. IP 397
745	*N. asteroides* E. Originally isolated from a Swedish patient (Bergström *et al.* 1966)
746	*N. farcinica* 378. Originally isolated from cattle at the Laboratoire élevage Curasson, Dakar. ATCC 13781. IP 378
749	*N. farcinica* 736. Originally isolated from cattle with farcy at the Farcha Laboratory, Fort-Lamy, Chad. IP 736.
750	*N. farcinica* 740. Originally isolated from cattle with farcy at the Farcha Laboratory, Fort-Lamy, Chad. IP 740
751	*N. farcinica* 744. Originally isolated from cattle with farcy at the Farcha Laboratory, Fort-Lamy, Chad. IP 744
752	*N. asteroides* RG 3318. Originally isolated from cattle with farcy by Nocard (Waksman, 1957). Type strain of *N. farcinica*, authentic co-type, see Sneath & Skerman (1966). ATCC 3318. NCTC 4524
753	*N. asteroides* RG 3399. Originally isolated by Dr C. P. Fitch, New York, U.S.A. (Waksman, 1957). ATCC 3399
787	*N. blackwellii* McCL 81. Originally isolated from a foal (Erikson, 1935). ATCC 6846. NCTC 630. RG 503
809	*N. asteroides* RG 727. Originally isolated from sputum from a patient named Jackson by Dr W. M. Bowman, Georgia State Department of Public Health. Proposed as reference strain of *N. asteroides* (Sneath & Skerman, 1966).

* Cultures labelled SSC were received from Dr H. C. Engbæk, Statens Seruminstitut, Copenhagen, Denmark; those labelled PH were received from Dr P. Holm, Statens Seruminstitut, Copenhagen, Denmark; those labelled RG were received from Dr Ruth Gordon, Institute of Microbiology, Rutger's State University, New Brunswick, New Jersey, U.S.A.; those labelled G were received from Dr Guilhon, Ecole Vétérinaire, Alfort, France; the one labelled E was received from Dr L. Edebo, Department of Bacteriology, University of Uppsala, Sweden, and the one labelled McCl from Dr N. M. McClung, Department of Bacteriology, University of Georgia, Athens, Georgia, U.S.A.

ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; EM, Dr C. W. Emmons, National Institute of Allergy and Infectious Diseases, Bethesda, Md., U.S.A.; IP, Institut Pasteur, Paris, France; NCTC National Collection of Type Cultures, London, England.

Experimental design. The data presented here were drawn from a series of 16 studies. In most cases the experimental design of the single studies was one of those described previously (Magnusson *et al.* 1961), 5 strains being compared with each other, or each of 4 strains compared with each of 4 other strains. However, results were also drawn from one study in which 10 strains were compared with each other, and from another in which each of 9 strains was compared with each of 9 other strains. In the two latter studies only the highest concentration of each sensitin was used.

Reading of reactions. The reactions were read after 24 hr and the longitudinal and transverse diameters of erythema recorded in millimetres. Each reaction was read by two readers. To avoid bias in reading the reactions, the dilutions were labelled by code.

Calculation of specificity differences (spd). Using averages of the reading of both readers, the difference in specificity of two sensitins was calculated as the difference between the homologous and the heterologous reactions as follows: spd $= (A_a + B_b) - (A_b + B_a)$, where A_a is the average homologous reaction obtained with sensitin A in animals sensitized with strain a. A_b is the average heterologous reaction obtained with sensitin A in animals sensitized with strain b, B_b and B_a denote the average homologous and heterologous reactions with B sensitin. Examples of the calculation have been published previously (Magnusson, 1961).

Each spd value reported below is the average of four determinations. In previous studies (Magnusson, 1961, Magnusson *et al.* 1961) where the same technique has been used, the standard deviation was 1·1–1·4 mm. for spd $\leqslant$ 3 mm. and 2·0–2·2 mm. for spd $>$ 3 mm.

RESULTS

The results of calculation of the specificity differences (spd) are shown in Table 2; sensitins showing spd $\leqslant$ 3 mm are grouped together. The largest group contains 10 sensitins, including preparations from strain 752, which presumably originates from Nocard, and four other strains (749, 750, 751, 753) designated *Nocardia farcinica* as received. Sensitins from three strains (84, 85, 787), isolated from a cow, a dog and a

Table 2. *Specificity differences of sensitins*

The table shows the differences (in mm.) between homologous and heterologous reactions to intradermal tests in guinea pigs sensitized with the strains used for preparation of the sensitins. Italicized figures were published previously (Magnusson, 1962).

Strain	Sensitin: RS 84	RS 85	cf 752	cf 753	cf 749	cf 750	cf 751	cf 650	cf 745	cf 787	RS 80	cf 678	cf 746	cf 677	RS 81	cf 809	RS 117
*RS 84 *Nocardia asteroides*	—	0	2	1	0	−1	0	3	3	2	4	12	15	13	*13*	3	*14*
RS 85 *N. asteroides*	0	—	2	3	—	—	—	−2	2	—	*5*	—	—	—	*11*	—	—
†cf 752 *N. farcinica*	2	2	—	3	0	1	3	—	3	1	2	11	—	6	11	6	—
cf 753 *N. farcinica*	1	3	3	—	1	0	0	—	—	2	3	14	—	11	13	6	—
cf 749 *N. farcinica*	0	—	0	1	—	−2	1	—	—	2	3	14	—	11	10	7	—
cf 750 *N. farcinica*	−1	—	1	0	−2	—	2	—	—	3	3	16	—	14	12	11	—
cf 751 *N. farcinica*	0	—	3	0	1	2	—	—	—	—	2	—	—	—	10	9	—
cf 650 *N. asteroides*	3	−2	—	—	—	—	—	—	—	—	4	—	—	—	10	—	—
cf 745 *N. asteroides*	3	2	3	—	—	—	—	—	—	—	2	—	—	—	12	—	—
cf 787 *N. blackwellii*	2	—	1	2	2	3	—	—	—	—	—	—	—	—	—	—	—
RS 80 *Nocardia* sp.	4	*5*	2	3	3	3	2	4	2	—	—	13	—	28	*17*	9	*18*
cf 678 *N. farcinica*	12	—	11	14	14	16	—	—	—	—	13	—	−2	9	10	14	11
cf 746 *N. farcinica*	15	—	—	—	—	—	—	—	—	—	—	−2	—	10	—	—	—
cf 677 *N. farcinica*	13	—	6	11	11	14	—	—	—	—	28	9	10	—	21	12	14
RS 81 *N. asteroides*	*13*	*11*	11	13	10	12	10	10	12	—	*17*	10	—	21	—	9	*13*
cf 809 *N. asteroides*	3	—	6	6	7	11	9	—	—	—	9	14	—	12	9	—	9
RS 117 *N. brasiliensis*	*14*	—	—	—	—	—	—	—	—	—	*18*	11	—	14	*13*	9	—

* RS = purified sensitin. † cf = heat-sterilized culture filtrate.

foal respectively, are also included in this group. This also applies to the sensitin of strain 745, isolated from man, and to strain 650, of unknown source.

Sensitins from three other strains (678, 746, 677) designated *Nocardia farcinica* on receipt cannot be included in this group (spd = 6–16 mm), but two of them (cf 678, cf 746) can be grouped together (spd = −2 mm).

Sensitin from strain 80 shows spd ⩽ 3 mm. when compared with 6 of the sensitins in the main group and spd = 4–5 mm. when compared with the sensitins of three strains in this group. (The spd between RS 80 and RS 84 reported previously (Magnusson, 1962) is not correct, because of an error in calculation.) Therefore this sensitin 80 cannot be included in the main group by the criterion mentioned above.

Sensitins from all 14 strains show spd values varying from 3 to 21 mm. when compared with sensitins of *Nocardia asteroides* strain 81, *N. asteroides* strain 809 and *N. brasiliensis* strain 117, and therefore none of these preparations are grouped together.

The spd between sensitins of *Nocardia asteroides* strain 81 and *N. asteroides* strain 809 is 9 mm.

Classification of the strains on the basis of sensitin specificity

On the basis of the specificity differences of the sensitins and with the criteria for their utilization in classification used earlier (Magnusson *et al.* 1961; Magnusson, 1962), the strains can be classified as follows: strains 84, 85, 650, 745, 749, 750, 751, 752, 753, 787 as *Nocardia farcinica*; strains 678 and 746 as *Nocardia* sp.; strain 677 as *Nocardia* sp. and strain 80 as *Nocardia* sp.

DISCUSSION

The relationship between the strains found in this work by an immunological method can be supported by only a few results obtained by other methods. However, the method used here has given reproducible and reliable results in studies of more than 250 strains of mycobacteria. The relationships found between mycobacteria by this method have been confirmed by observations of the cultural, physiological and biochemical characteristics of those strains made in other laboratories. Identification at species level of a test strain by this method has been possible when an spd value of < 3 mm. between sensitin of a reference strain belonging to a known species and the test strain has been found. SPD values of 3 or 4 mm. have generally been considered inconclusive. These observations with mycobacteria have contributed to the following evaluation of the present results.

With one exception (strain 80) clear results have been obtained with all the 17 strains. Strain 80 has been classified as *Nocardia asteroides* (Dr Ruth Gordon, personal communication).

Ten of the strains, including the type strain of *Nocardia farcinica* (752) and 4 other strains (749, 750, 751, 753) previously identified as *N. farcinica*, form one group. Strains 749, 750, 751 were isolated from cattle with farcy and identified on the basis of their morphology and biochemical properties (Perpezat, Mariat, Destombes & Thomé, 1963). The five other members of this group are strains 84, 85, 650, 745, 787.

Strains 84 and 85 were identified as *Nocardia asteroides* when originally isolated from the placenta of a cow and from a dog, respectively (Fey, Holm & Teuscher, 1954).

Strain 650 was given by Professor N. F. Conant, North Carolina, U.S.A. Although its original source could not be traced, it cannot be the same isolate as any of the strains labelled *Nocardia farcinica*, since these strains were not kept in Conant's laboratory at the time when strain 650 was sent to the National Collection of Type Cultures, London (Professor N. F. Conant, personal communication).

Strain 745 originated from a human patient (Bergström, Edebo, Fors & Tegner, 1966). In the report describing its isolation, the strain was repeatedly referred to as *Nocardia asteroides*, although it was also mentioned that it had been identified as *N. farcinica* by the present writers.

Strain 787 has been classified as *Actinomyces blackwellii* by Erikson (1935); it is characterized by its medium growth consistency, good formation of aerial mycelium with frequent angular branching, no soluble pigment on protein media, no liquefaction of gelatine and no action on milk (Erikson, 1935). This description is based on the study of the properties of a single strain (787). Schneidau & Shaffer could neither distinguish this strain from 6 strains designated *Nocardia asteroides* in cultural studies (1957) nor by use of a slide agglutination test (1960).

Strains 650 and 787 have been classified as *Nocardia asteroides* by Gordon & Mihm (1957). The same applies to strains 84 and 85 (Dr Ruth Gordon, personal communication). As mentioned in the Introduction, Gordon & Mihm (1959) could distinguish neither the type strain of *N. farcinica* (strain 752) nor strain 753 from a series of strains which those authors called *N. asteroides*. In addition to strains 81 and 809 which were clearly distinguishable in the present work from the group of 10 strains (spd = 3–13 mm.) and from each other (spd = 9), the latter also included others which can be distinguished from this group, represented by strain 84 (unpublished data). These observations definitely do not support the opinion expressed by Gordon & Mihm (1962) that *N. farcinica* and *N. asteroides* are two different names for the same species.

It would appear justifiable to classify the 10 strains in the largest group only as *Nocardia farcinica*. In consequence, *N. blackwellii* and *Actinomyces blackwellii* are subjective synonyms of *N. farcinica*. The three remaining strains labelled *N. farcinica* (677, 678, 746) are quite distinct from the group of 10 strains in the present study. On the basis of the present findings, the writers consider strains 678 and 746 form one separate species and 677 another.

Six strains classified in this work as *Nocardia farcinica* (84, 85, 650, 752, 753, 787) have been examined by the methods described by Gordon & Mihm (1962). All of them grew at 50° and four (84, 85, 650, 787) were able to ferment rhamnose (Dr Ruth Gordon, personal communication). Among 8 other strains which were classified as *N. asteroides* according to Gordon & Mihm's criteria and which, according to the present method, are distinguishable from *N. farcinica* (unpublished data), none showed both the above-mentioned characteristics (Dr Ruth Gordon, personal communication). Of 142 strains classified as *N. asteroides*, only 21 % grew at 50° (Gordon & Mihm, 1962). The strains classified as *N. farcinica* in the present work (except strain 749) have a higher growth rate at 45° than other strains classified as *N. asteroides* by Gordon & Mihm's criteria. It may thus be possible to demonstrate characteristic physiological and biochemical features of *N. farcinica* strains and to differentiate between that species and *N. asteroides* by conventional methods also. However, further studies are required.

It was reported earlier that 29 out of 30 strains of *Nocardia asteroides* were urease-positive, in contrast to 14 strains of *N. farcinica*, including 749, 750 and 751 which were urease-negative (Mariat, 1963). Strains 752 (the type strain of *N. farcinica*) and 753 are both urease-positive (unpublished observation). Thus the urease reaction may not always be of help in the distinction between *N. farcinica* and *N. asteroides*.

According to Waksman (1957), strains of *Nocardia farcinica* are transmissible to guinea pigs, cattle and sheep but not to rabbit, dogs or horses. However, two of the strains identified as *N. farcinica* in the present work (84, 85) were reported to be transmissible to rabbits (Fey *et al.* 1954). As mentioned earlier, one of these two strains was isolated from a dog, and strain 787, which has also been identified as *N. farcinica* in the present work, was originally isolated from a foal (Erikson, 1935). Therefore, according to the present findings, the description of *N. farcinica* given by Waksman (1957) and based on two strains only, should apparently be revised as regards the pathogenicity of the species. It is remarkable that strain 745, identified as *N. farcinica* in the present work, was isolated from a human patient (Bergström, *et al.* 1966). This is apparently the first known case of human infection caused by *N. farcinica*. Other such cases may be found, particularly where the causal agent has been identified as *N. asteroides* and where the isolate has been rhamnose-positive and able to multiply at 50°. Strains of *N. farcinica* isolated from human patients may also be identified among the strains which could not be distinguished from *N. blackwellii* in the study reported by Schneidau & Shaffer (1960).

The writers are most grateful to Dr L. Edebo, Department of Bacteriology, University of Uppsala, Sweden; Dr Ruth Gordon, Institute of Microbiology, Rutgers University, New Brunswick, New Jersey, U.S.A.; and Dr N. M. McClung, Department of Bacteriology, University of Georgia, Athens, Georgia, U.S.A., for providing cultures.

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