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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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

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

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

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.) as well as attempting to convey their meaning to their readers.

(6) Editors do not alter author's typescripts except to increase clarity and conciseness. If an editorial alteration changes an author's meaning one implication is that it was expressed ambiguously. When an editor can grasp the meaning of a sentence unequivocally it may be assumed that anyone can.

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Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in *J. gen. Microbiol.* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

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The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

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

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Some Properties of a Sporulating *Bacillus subtilis* Mutant Containing Heavy DNA

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(*Accepted for publication* 28 *February* 1969)

SUMMARY

A mutant (Cbl-1) was isolated from *Bacillus subtilis* 168 wild type (*wt*) sporulating cultures grown on nutrient agar. This mutant differs from the parental strain as follows. (1) In nutrient broth the mutant excretes a substance which kills the *wt* cells. Therefore when grown in a mixed culture (Cbl-1 plus *wt*), this property leads to a take-over pattern by the Cbl-1 mutant, and the spores obtained from such a culture are entirely of the mutant type. (2) The DNA of the Cbl-1 strain has a buoyant density in CsCl of 1·714 g./cm.^{3} while that of the normal *B. subtilis* 168 is 1·703 g./cm.^{3} although both strains have a G+C content of 42 moles %. An explanation for the higher buoyant density of the Cbl-1 DNA has not yet been found. In addition to the base ratio data, transformation and transduction experiments indicate that Cbl-1 is a mutant of *B. subtilis* 168.

INTRODUCTION

The interaction between populations in bacterial cultures is a well-known phenomenon which leads frequently to unpredictable results. The phenotypic expression and the proportions of types of bacteria in a microbial population is strictly controlled through selective mechanisms by the interaction of the bacteria in a given environment.

One such typical example is the rough–smooth variation of *Brucella abortus* (Braun *et al.* 1951). The proportions of these two types of cells in the culture is regulated by the concentration of alanine. As the smooth inoculum grows it excretes alanine, which stimulates its further growth but not that of the rough cells. As a result a progressive selection of the smooth cells ensues, so that during the stationary phase they are the predominant type in the population.

Another example of cellular interaction was recently reported by Meers & Tempest (1968). Their studies concern the influence of extracellular products on the behaviour of mixed microbial populations in magnesium-limited cultures. The ability of *Bacillus subtilis* and *Bacillus megaterium* to outgrow each other or outgrow yeast in a magnesium-limited medium depends on the concentration of a specific extracellular factor produced in the Bacillus cultures.

The experiments presented here demonstrate an unexpected predominance of a mutant of *Bacillus subtilis* 168 when grown in a mixed culture with the parental

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wild-type strain. The results to be described provide also an explanation of the presence of 'heavy' DNA in the spores of *B. subtilis*, previously reported from this laboratory (Halvorson, Szulmajster, Cohen & Michelson, 1967). This spore DNA was reported to differ from that isolated from the vegetative forms of this organism by its higher buoyant density in caesium chloride and by a higher *Tm*, although the base composition of these two DNAs was found to be identical. Although a biological role was not assigned to this heavy 'spore' DNA (Halvorson *et al.* 1967), the reported data was suggestive of a difference between spore DNA and vegetative cell DNA. This impression was also supported by similar findings of Douthit & Halvorson (1966) in *B. cereus*. However, this interpretation seems to be erroneous, at least in *B. subtilis*, in the light of the unexpected physiological behaviour and properties of a spontaneous mutant found in the same *B. subtilis* cultures, used in the previous work (Halvorson *et al.* 1967). Thus the 'heavy' DNA appears to be that of the spontaneous Cbl-1 mutant rather than specific for spores.

METHODS

Organisms. Derivatives of *Bacillus subtilis* strain MARBURG were used throughout these studies. The genotype source and method of isolation are shown in Table 1. To minimize heterogeneity, all of the markers studied were introduced into *B. subtilis*

Table 1. *Strains used in this study*

Strain	Genotype*	Origin	Parent strain	Source
B. subtilis 168	*wt*			
B. subtilis 168M	*trpC2*	—	—	C. Anagnostopoulos
Cbl-1	*wt*†	Spontaneous	168	
Cbl-1 Ery^r	*ery-1*	Spontaneous	Cbl-1	
BR19	*trpC2*, *hisA1*	—	168	B. Reilly
BR85	*trpC2*, *argC4*	—	168	J. Marmur
BR123	*trpC2*, *argO3*	—	279	M. Kelly
BR151	*trpC2*, *lys-3*, *metB10*	—	279	M. Kelly
B. subtilis I$^-$ Thy	*trpC2*, *thyA*, *thyB*	Spontaneous	168	J. Farmer
BY12	*gtaA12*	Spontaneous	—	—
BR290	*gtaB290*	Spontaneous	—	B. Reilly

* Abbreviations: *wt*, wild type; *trp*, tryptophan; *ery^r*, erythromycin-resistant; *his*, histidine; *arg*, arginine; *lys*, lysine; *met*, methionine; *thy*, thymine; *gtaA* and *gtaB* are stains blocked at different steps in glucosylated teichoic acids synthesis (Young, 1967).

† This strain has an albinose phenotype.

168 by Reilly (Young, Smith & Reilly, to be published). The Cbl-1 strains were isolated from several aged spore stock cultures of *B. subtilis*, 168 and SMY. These strains are the same as those used previously (Halvorson *et al.* 1967). Evidence will be presented that Cbl-1 is most likely a mutant of the 168 strain. This organism can hardly be detected in *B. subtilis* populations when plated on minimal agar media or in overnight cultures on complex media. After 2–4 days of growth on nutrient medium, the small white mutant colonies are readily distinguished from the larger dark brown *wt* colonies.

An erythromycin-resistant derivative from the Cbl-1 mutant was isolated by plating 0·1 ml. of a heavy spore suspension (about $1-2 \times 10^8$ spores/ml.) after heating

10 min. at 80°, on nutrient broth agar (Difco) containing 1 μg. erythromycin/ml. After 2 days at 37°, eleven colonies which still formed spores (Spo^+) were isolated from 25 plates. These clones retained albino phenotype and were erythromycin-resistant.

Culture media and bacterial assays. The sporulation medium used in most of the experiments has been described previously (Kerjan, Marchetti & Szulmajster, 1967). In some experiments the nutrient broth was replaced by Bacto-peptone (Difco). Each new batch of the above nutrients was checked to determine whether it provided a high rate of sporulation (at least 80% of the population in 24 hr) before serial experiments were undertaken.

Liquid cultures were always grown in Fernbach flasks containing a volume of medium not exceeding one-tenth of the volume of the flask. Cultures of 250 ml. were usually inoculated with exponential cultures. The inocula of the parent strain were prepared by inoculating 10 ml. of medium with spores from agar slopes. For Cbl-1, cells grown for 18 hr on Tryptose Blood Agar base (TBAB, Difco) were used instead of spores. All the cultures were incubated at 37° with vigorous aeration. Growth was followed by determining the extinction at 650 mμ in a Zeiss spectrophotometer.

Viable counts were made by plating the organisms on nutrient broth agar (Difco) after suitable dilutions in sterile 0·05 M phosphate buffer (pH 7·0). The number of spores was counted after heating the suspension for 10 min. at 80°. In some experiments the number of spores was determined in a Petroff–Hausser counting chamber with a phase-contrast microscope.

Isolation of DNA. DNA from vegetative forms was prepared according to Marmur (1961) and from spores by the method described previously (Halvorson *et al.* 1967).

Sedimentation equilibrium measurement in CsCl was performed in the Spinco Model E ultracentrifuge (Meselson, Stahl & Vinograd, 1957) using ultraviolet absorption photographs scanned with a Joyce–Loebel recording micro densitometer. The density of DNA was calculated by using the 2 C phage DNA (a gift from Dr P. May) as a reference.

Base ratios were determined as previously described (Halvorson *et al.* 1967).

Transduction. Modification of the procedures of Takahashi (1961), and Hoch Barat & Anagnostopoulos (1967) were used to prepare lysates and obtain transduction (Young, Smith & Reilly, to be published). After transduction of a motile varient of BR 19 (carrying *hisA1* and *trp-2*) the His^+ clones were picked and subsequently replicated on TBAB plates in the absence and presence of phage ø 25 to test for phage resistance.

Transformation. The procedures for preparing competent bacteria were similar to those described previously (Young & Spizizen, 1961) with the exception that bacteria were grown for 5 hr in medium 1 and 65 min. in medium 2. Auxotrophic requirements were satisfied by adding 50 μg./ml. of the necessary amino acids.

Transformation was also achieved by treating the recipient bacteria at T_3 (3 hr after exponential growth) with donor DNA for 30 min. and terminating the reaction by adding 100 μg. (0·1 ml.) of pancreatic deoxyribonuclease (Worthington) (Bott & Wilson, 1967). Shaking was continued for 15 min. and the cells centrifuged down. Suitable dilutions of the cells were then made in minimal medium and plated either on Spizizen minimal medium agar plus or minus tryptophan the required amino acid or on nutrient broth agar plus or minus erythromycin. According to Takahashi (1965)

the erythromycin-resistant transformants were plated in 10 ml. of melted nutrient broth agar (Difco), kept at 45°, left for 3 to 4 hr at 37°, at which time the plates were overlaid with 10 ml. of the same medium containing erythromycin 2 μg./ml. The numbers of erythromycin-resistant colonies were counted after 2 days. All the recipient strains utilized for transformation contained the mutant *trpC2* locus as well as other mutations (Table 1). Thus, it was possible to compare all data to the frequency of transformation for *trp-2* markers.

RESULTS

Physiological behaviour of the Cbl-1 mutant

Figure 1 shows that the 168 strain of *Bacillus subtilis* or an artificial mixture of this strain and the mutant Cbl-1 (1:1 ratio) initiated growth immediately at an exponential rate. Under the same conditions growth of the Cbl-1 strain started with

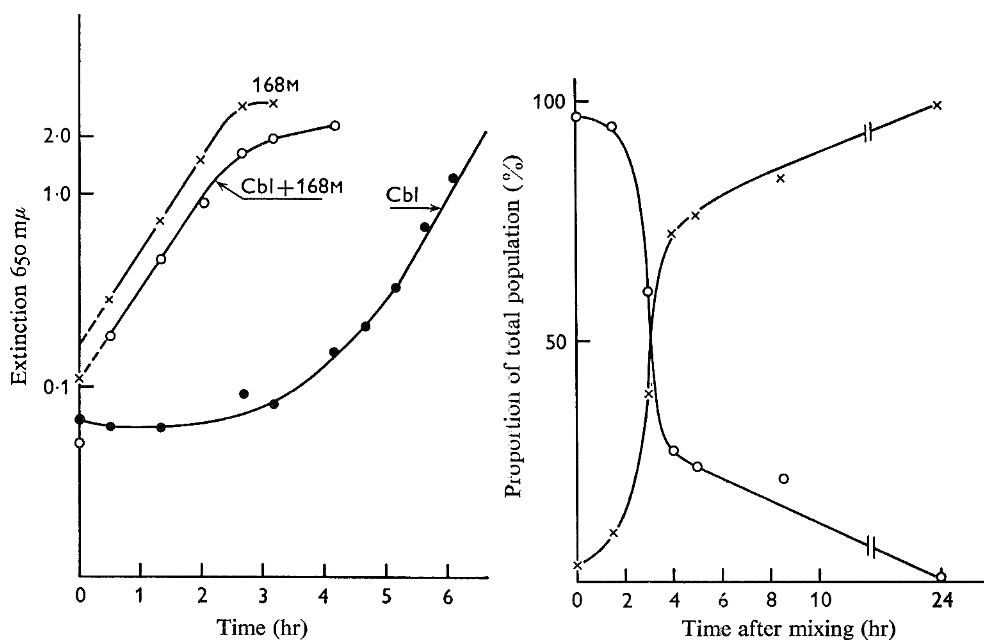

Fig. 1. Growth of *Bacillus subtilis* 168M and Cbl-1 strains in separate or in mixed cultures. Three Erlenmeyer flasks (250 ml.), each containing 25 ml. nutrient broth supplemented with the usual metals, were inoculated with exponentially growing inocula from: (1) 168 *wt*, (2) Cbl-1, and (3) 168M+Cbl-1 (1:1). Growth was followed by measuring the O.D. (650 mμ).

Fig. 2. Development of 168M and Cbl-1 populations in an artificial culture. ×—×, Cbl-1 at the initial concentrations of 2×10^6 bacteria/ml. O—O, *wt* at 6×10^7 cells/ml.

a lag which lasted about 4 hr. In some experiments the lag was as long as 5 hr. Growth in the mixed culture was due to the 168 strain as observed by plating samples at different time intervals on nutrient broth agar after suitable dilutions.

Figure 2 shows the proportion of the two types of cell in a mixed culture. The mixture was made with exponentially growing cells of the two cultures in proportions shown in the figure at time 0. Cells of each type are readily distinguished by their

phenotype on nutrient agar. After 35 hr of incubation the surviving *wt* organisms were almost entirely spores.

As shown in Fig. 3 the ability of Cbl-1 to take over the population depended on the relative amounts of each bacterial type in the culture after mixing the two populations.

Development of spores in separate and mixed cultures of wt and Cbl-1. Three flasks, *a*, *b* and *c*, each containing 25 ml. of nutrient broth (Difco) were inoculated with equal proportions of exponentially growing cells of (*a*) strain 168 M, (*b*) Cbl-1 and

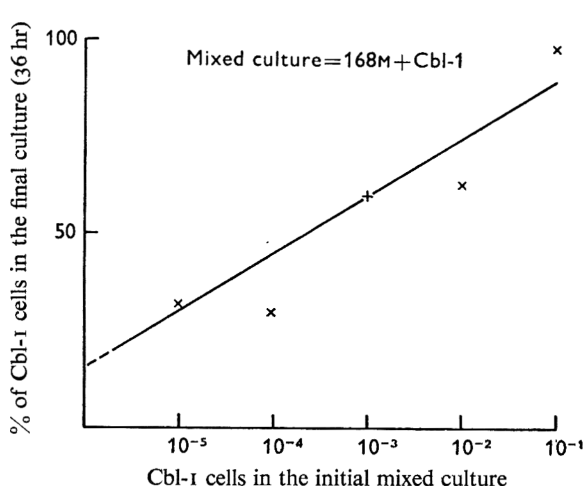

Fig 3

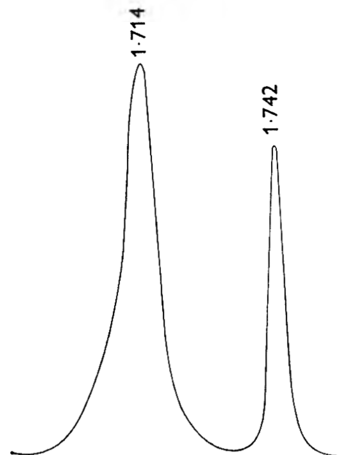

Fig. 4

Fig. 3. Dependence of the number Cbl-1 cells in a final mixed culture on the Cbl-1/168M ratio present in the initial culture after mixing.

Fig. 4. Buoyant density of Cbl-1 DNA. Micro densitometer tracings of samples equilibrated in a CsCl density gradient formed by centrifugation at 42,040 rev./min. at 20°. The band on the right is the standard 2 C phage DNA.

Table 2. *Evolution of spore formation in separate and mixed culture of 168 M and Cbl-1 strains*

				168M+Cbl-1	
		168M	Cbl-1	168M	Cbl-1
T_0	C	$1{\cdot}6\times10^8$	$7{\cdot}7\times10^7$	1×10^8	7×10^7
	S	—	—	—	—
T_5	C	$2{\cdot}6\times10^8$	$3{\cdot}2\times10^8$	$6{\cdot}5\times10^7$	6×10^7
	S	$3{\cdot}5\times10^6$	$7{\cdot}5\times10^6$	$< 10^5$	$3{\cdot}5\times10^6$
T_7	C	$3{\cdot}5\times10^8$	$2{\cdot}8\times10^8$	7×10^7	7×10^7
	S	$7{\cdot}5\times10^7$	7×10^7	$< 10^5$	2×10^7
T_9	C	$3{\cdot}6\times10^8$	$2{\cdot}5\times10^8$	3×10^7	1×10^8
	S	$1{\cdot}2\times10^8$	1×10^8	2×10^5	$4{\cdot}5\times10^7$
T_{24}	C	$2{\cdot}9\times10^8$	$3{\cdot}1\times10^8$	5×10^6	$1{\cdot}2\times10^8$
	S	$2{\cdot}2\times10^8$	$3{\cdot}5\times10^8$	$< 10^5$	1×10^8
T_{40}	C	3×10^8	$2{\cdot}9\times10^8$	$< 10^5$	$9{\cdot}5\times10^7$
	S	$3{\cdot}5\times10^8$	3×10^8	$< 10^6$	$1{\cdot}2\times10^8$

C = bacteria before heating. S = bacteria after heating 10 min. at 80° C. (spores). T_0 designates the end of exponential growth; T_5, T_7 etc., indicate time in hr after T_0.

(*c*) an equal volume of *wt*+Cbl-1. Growth was followed by increase in extinction and by plating samples on agar plates in suitable dilution at different time intervals. T_0 (the end of exponential growth) was normalized for the three cultures by taking into account the 5-hr lag phase observed with the mutant strain in this experiment. The experiment shows (Table 2) that sporulation of the bacterial populations of the two strains in separate cultures occurs at the normally observed rate in nutrient broth medium. This is also true for the Cbl-1 strain in the mixed culture where the sporulation rate of the Cbl-1 bacteria was comparable to that in the corresponding separate culture. On the contrary, sporulation of the wild-type population in the mixed culture was extremely low throughout the entire incubation period and these bacteria were continuously lysing. Consequently, after 24 hr, almost the entire bacterial population was composed of Cbl-1 spores.

Table 3. *Effect of 168/Cbl filtrate on 168 M cells*

	Cbl-1 filtrates from cultures:	Cells*	Spores*
168M bacteria, harvested at T_3 and transferred to:	Exponential	105	103
	T_0†	85	15
	T_3	14·5	0·60
	T_7	1	0·65
	T_{24}	2·2	0·45
Control 168M bacteria at T_3 transferred to:	168M filtrate from a culture at T_3	100‡	100

* Relative values after 24 h.
† The end of exponential growth is designated as T_0; T_3, T_7, T_{24}, etc., indicate time in hr after T_0.
‡ In this experiment 100 is equal to $1·9 \times 10^8$ bacteria and to $1·8 \times 10^8$ spores.

Effect of Cbl-1 filtrates

It appeared that the Cbl-1 strain was excreting a substance toxic to and perhaps lytic for the wild type. Although the nature of this substance is not as yet known it was of interest to determine the time during the growth cycle when the excretion takes place. For this purpose sterile (Millipore) filtrates were prepared from a Cbl-1 culture at various stages of growth. These filtrates were kept overnight at room temperature. A 100 ml. culture of *wt* was grown, the bacteria harvested at T_3 and suspended in 2 ml. of fresh nutrient broth. Each sterile filtrate was inoculated with 0·2 ml. of the 168 suspension and incubated at 37° for 24 hr with vigorous shaking. In the control experiment the 168 suspension was transferred to its homologous filtrate. Viable bacteria were counted by plating before and after heating (spores) on nutrient broth agar as described in 'Methods'.

A typical experiment is summarized in Table 3. The filtrate prepared from exponential Cbl-1 bacteria supported growth and had no effect on the sporulation of the *wt*. When the 168 cells were inoculated in a T_0 filtrate, most of the bacteria remain viable but only about 15% of them formed spores. The most dramatic effect was observed when wild-type bacteria were inoculated in a Cbl-1 filtrate prepared from T_3 or later cultures. Very few bacteria remain viable and consequently very few spores were formed. A change in the number of bacteria or spores was not observed in the control which had not been transferred to filtrates of Cbl-1. These results suggest that the substance lysing or killing the wild-type bacteria is excreted by the Cbl-1 bacteria into the medium

during the stationary phase of growth. It was also observed that the Cbl-1 filtrate had no effect on bacteria of *Bacillus cereus*, *B. megaterium*, *B. licheniformis* or *Escherichia coli* harvested at the post-logarithmic growth phase.

Buoyant density of Cbl-1 DNA in CsCl

The physiological experiments cited above directed our attention to the earlier observations of the appearance of a satellite band of DNA in sporulating cultures of *Bacillus subtilis* 168, which differed from that found in the vegetative forms by its higher buoyant density in CsCl equilibrium centrifugation and by its higher *Tm* (Halvorson *et al.* 1967).

Figure 4 shows the buoyant density in CsCl equilibrium centrifugation of the DNA isolated either from the vegetative forms or from the spores of the Cbl-1 mutant strain: $e = 1{\cdot}714$ g./cm.^{3}. This is precisely the density previously reported for the 'heavy' DNA found in the *Bacillus subtilis* spores. In addition, the base ratio (Table 6) of the Cbl-1 DNA (vegetative or spore) was found to be identical to that reported for the 'heavy' DNA (G+C = 42 to 43 %) (Halvorson *et al.* 1967).

Genetic homology of Cbl-1 and B. subtilis 168

Is Cbl-1 a contaminant or a mutant derived from *Bacillus subtilis* 168? Three different types of experiments support the contention that Cbl-1 is a mutant of *B. subtilis* 168.

Table 4. *Transformation of Bacillus subtilis 168 auxotrophs with DNA B. subtilis wt and Cbl-1*

	Transformants/ml. in recipients*					
	BR151			168 thy⁻		168M
Donor DNA	*lys-3*	*trp-2*	*metB10*	*thy*	*trp-2*	*Ery^r*
168 *wt*	$7{\cdot}0 \times 10^5$	$6{\cdot}7 \times 10^5$	$7{\cdot}0 \times 10^5$	$3{\cdot}0 \times 10^4$	$2{\cdot}3 \times 10^4$	ND
Cbl-1 *Ery^r* (vegetative)	$3{\cdot}3 \times 10^2$	$1{\cdot}3 \times 10^3$	$1{\cdot}3 \times 10^3$	ND	ND	$5{\cdot}2 \times 10^3$
Cbl-1 (spore)	10^2	$< 10^2$	$< 10^2$	$2{\cdot}1 \times 10^3$	$< 10^2$	ND

* Viable count varied from 9×10^7 to 2×10^8 in these experiments with all the recipient strains used. ND: not determined. In these experiments the concentration of *wt*, Cbl-1 vegetative and spore DNA was 1, 0·5 and 0·1 μg./ml. respectively.

Firstly, the transformation experiments shown in Table 4 demonstrate that the DNA isolated from Cbl-1 is capable of transforming mutants of *Bacillus subtilis* 168, although with a lower efficiency than *wt* DNA. It can also be seen in that Table that when the *thy*, *trp* auxotroph of *B. subtilis* 168 was used, transformation by the Cbl-1 spore DNA was limited only to the *thy* phenotype. At present no explanation can be given to this surprising observation.

Secondly, traits from Cbl-1 can also be transferred to strains of *Bacillus subtilis* by PBS-1 mediated transduction. In these experiments lysates of PBS-1 were prepared on motile vegetative populations of Cbl-1. These lysates were used to transduce three strains of *B. subtilis* 168, BR19, BR85 and BR151. Successful transduction of the mutant loci in these strains was obtained in all experiments. As shown in Table 5, the locus-governing phage resistance in Cbl-1 is linked to *hisA1*. The percentage

co-transfer of Pha^r with *hisA1* is similar to that observed previously with class A and class B phage-resistant mutants of *B. subtilis* 168 (Young, Brown & Reilly, 1968).

Thirdly, as shown in Table 6, the base ratio of the Cbl-1 spore or vegetative form DNA is consistent with that obtained by other authors for *B. subtilis* 168 (Schildkraut, Marmur & Doty, 1962).

Table 5. *Linkage of phage-resistance markers to his A1* in PBS-1 transduction*

Donor (PBS-1 lysates)	Phenotype of recombinants	
	His$^+$	Pha^r
gtaA12	400	245
gtaB290	600	267
Cbl-1	400	201

* Recipient BR19 (*hisA1*, *trp-2*).

Table 6. *Base ratios of Cbl-1 spore and vegetative form DNA*

Source of DNA	A	G	C	T	G+C (%)
Vegetative forms	28·2	21·8	21·7	28·3	43·5
	29·1	20·9	(20·9)	(29·1)	41·8
Spores	28·3	21·6	21·0	29·3	42·6
Spores*	28·2	21·9	20·5	29·4	42·4

* From Halvorson, Szulmajster, Cohen & Michelson (1967).

DISCUSSION

The results presented here force a reinterpretation of the previously reported work from this laboratory (Halvorson *et al.* 1967), which led to the suggestion that *Bacillus subtilis* spores contained a 'heavy' DNA. In fact the spores from which the 'heavy' DNA was isolated derived from the Cbl-1 mutant present in the cultures. We have shown here that through its physiological properties the Cbl-1 strain, when present in a culture with 168 strain in relatively small numbers, may lead to a complete inversion of the bacterial type in the population.

Furthermore, because of the long lag of the Cbl-1 strain, these bacteria will be undetectable in such a mixed culture during exponential growth which will be dominated by the wild-type population (168 strain). Therefore, the DNA extracted from the bacteria during the latter period will have the properties of the normal *Bacillus subtilis* DNA with its known density in CsCl, e = 1·703 g./cm.^{3}. When exponential growth of the *wt* stops, the Cbl-1 mutant starts to develop (Fig. 1) and continues to grow partially at the expense of the *wt* which lyses. This lysis seems to affect the wild-type population during its entire presporulation period. This phenomenon leads rapidly to an increase of the proportion of Cbl-1 bacteria in the total population. As a result, the DNA extracted at this stage (T_5–T_6) will show, in CsCl gradient centrifugation, a satellite 'heavy' DNA band in addition to the main band characteristic of the normal *B. subtilis* DNA. Thus, a complete take-over by the Cbl-1 bacteria occurs in the culture and spores produced after 24 to 36 hr are derived predominantly from the Cbl-1 population. Consequently, if DNA is isolated from the dormant spores

of such a culture, 85 to 95 % of the DNA will have the density of 1.715 ± 0.002 g./cm.^{3}, characteristic of the Cbl-1 mutant. In addition, it has also been observed that DNA is extracted from the Cbl-1 lyophylized spores by the method described previously (Halvorson *et al.* 1967) with an efficiency 2 to 3 times higher than from those of *B. subtilis* 168.

Base ratio analysis, transformation and transduction experiments, all indicate that Cbl-1 is a mutant of *Bacillus subtilis* 168 and not a contaminant. The accumulation of sporulation mutants in old cultures of bacilli is a well-known phenomenon. This is explained by the fact that after sporulation is completed, autolysis of the sporangia provides a new nutrient supply for the non-committed bacteria present in the population (Michel, Cami & Schaeffer, 1968). It is quite conceivable that the new nutrient supply may, in some cases, also favour the selection of a particular mutant similar to that reported here. The Cbl-1 mutants, as already mentioned, can easily escape detection in overnight cultures plated on nutrient agar, which are generally used as inoculum for liquid media. It is therefore not surprising that a given number of unrevealed mutant cells are introduced in liquid cultures together with the wild-type bacteria.

The problem which still remains unsolved is the cause of the high density of the Cbl-1 DNA despite its base ratio being identical to the normal *Bacillus subtilis* DNA. Chemical analyses (P, N, amino acids, abnormal bases and metals) have so far given no answer to the question.

Another interesting and perhaps fundamental aspect of the Cbl-1 mutant concerns its resistance to phages which infect *Bacillus subtilis*. Previous experiments have demonstrated that glucosylated teichoic acid is essential for the adsorption of many of the phages which infect *B. subtilis* (Young, 1967). These phage-resistant strains can be subdivided into three classes. (1) Class A (*gtaA*) mutants are defective in UDPG:polyglycerol teichoic acid glucosyl transferase and are stimulated by galactose. (2) Class B (*gtaB*) mutants do not have any demonstrable defects in the enzymes involved in the biosynthesis of glucosylated teichoic acid yet fail to glucosylate this polymer. The growth of these mutants is not affected by galactose. (3) Class C (*gtaC*) mutants are defective in phosphoglucomutase and lyse in a medium containing galactose. Morphologically, Cbl-1 resembles *gtaB290*, a strain which grows slightly slower than *wt* and produces small colonies on nutrient agar (Reilly, 1965). It is possible that the primary defect in Cbl-1 is an alteration of the association of enzymes with the structural protein of the cytoplasmic membrane analogous to the mutants of Neurospora studied by Woodward (1968). Studies are in progress to determine the enzymic defect in Cbl-1 mutant. It may be important to note that the genes regulating surface structures such as flagella (Grant & Simon, 1968) glucosylation of teichoic acid (Young *et al.* 1968) and the rough–smooth variation of *B. subtilis* 168 (Young & Haywood, unpublished observations) are all located in one region of the chromosome.

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A Kinetic Study of the Growth of *Aspergillus nidulans* and Other Fungi

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SUMMARY

The growth kinetics of *Aspergillus nidulans*, *Mucor hiemalis* and *Penicillium chrysogenum* on solid media and in submerged culture were studied. Growth of *A. nidulans* colonies on solid medium can be divided into the four phases of lag, exponential, deceleration and constant growth rate. The growth kinetics of *A. nidulans* in submerged culture were similar to those commonly found for unicellular micro-organisms. The effects on colony radial growth rate, of glucose concentration, medium depth, oxygen partial pressure and temperature were studied. The radial growth rate of glucose-limited colonies of *A. nidulans* increased linearly with the log. of the initial glucose concentration. The effects of glucose concentration on internode length, hyphal density and hyphal diameter were also studied. At glucose concentrations above 1 % (w/v) there was an inverse relation between the radial growth rate of *A. nidulans* colonies and their peripheral hyphal density. The relations between colony radial growth rate (K_r) and germ tube specific growth rate (α_g) on solid media and specific growth rate in submerged culture (α_s) were investigated. Direct proportionality between K_r and α_s of *A. nidulans* was found by varying specific growth rate by temperature changes. The germ tubes of *A. nidulans* conidia grew exponentially at a rate which was about 2·3 times as great as the specific growth rate of the organism in submerged culture. The colony radial growth rates of the three moulds could not be used as a measure of their relative specific growth rates in submerged culture.

INTRODUCTION

Colony diameter and radial growth rate are frequently used as parameters of fungal growth in bioassays and physiological investigations. However, as pointed out by Pirt (1967), we lack detailed knowledge of the laws governing the response of microbial colonies to their environment.

When fungi are grown in submerged culture so that all nutrients are present in excess and growth inhibitors are not accumulated, the organism grows exponentially (Pirt & Callow, 1960) according to the equation:

$$dM/dt = \alpha M,$$

where M = mass of culture at time t and α is a constant known as the 'specific growth rate'. Integration of this equation gives:

$$\ln M - \ln M_0 = \alpha t,$$

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where ln = natural logarithm and M_0 = concentration of organism at zero time. The specific growth rate (α) is a direct measure of the growth rate of an organism and may be calculated from the expression:

$$\alpha = \ln 2/t_d,$$

where t_d is the doubling or mean effective generation time. Several workers have shown that filamentous fungi grow exponentially in submerged culture (Zalokar, 1959*a*; Pirt & Callow, 1960; Borrow *et al.* 1964). Swanson & Stock (1966), however, reported that the growth of *Microsporum quinckeanum* in submerged culture was not logarithmic. They suggested that filamentous fungi do not grow exponentially because individual hyphae grow at a constant rate. Mandels (1965), in a review of the 'Kinetics of fungal growth', supports this view, stating that '...growth is not autocatalytic in filamentous fungi'. However, although unbranched hyphae grow at a constant rate (Trinci & Banbury, 1967) mycelial hyphae are able to grow exponentially in submerged culture because they produce new growing points (i.e. branches) at intervals.

Colony diameter and radial growth rate are often used by mycologists to determine the optimum temperature for fungal growth (Brancato & Golding, 1953). This suggests that there may be a constant relationship between the colony growth rate of a fungus and its specific growth rate. No one, however, has determined whether such a relationship exists. Further, although it is well known from the work of Smith (1924) that spore germ tubes increase in length at an exponential rate, it is not known if the specific growth rates of germ tubes and of the organism in submerged culture are related. If a relationship exists, measurement of germ tube length might prove to be a convenient parameter of growth to use in bioassays and other work.

METHODS

Organisms. The organisms used were *Aspergillus nidulans* (BWB224 [Glasgow] recombinant ve y; this strain has a velvet morphology and yellow conidia); *Penicillium chrysogenum* WIS54-1255 (obtained from Biochemistry Department, University of Wisconsin, U.S.A.) and *Mucor hiemalis* (−) (Queen Elizabeth College strain Z14).

Medium. The composition of the medium (DAN) was (g./l.): D-glucose, 10; KH_2PO_4, 8·9; $Na_2HPO_4 12H_2O$, 8·9; $NaNO_3$, 6; EDTA, 0·6; $MgSO_4 7H_2O$, 0·25; $CaCl_2$, 0·05; $ZnSO_4 7H_2O$, 0·2; $MnSO_4 4H_2O$, 0·02; $CuSO_4 5H_2O$, 0·005; $FeSO_4 7H_2O$, 0·1; Na_2SO_4, 0·5; $NaMoO_4 2H_2O$, 0·005.

The basal medium was modified when *Mucor hiemalis* was grown by replacing $NaNO_3$ with 6 g. $(NH_4)_2SO_4$/l., decreasing the concentration of the phosphates by half and adding 0·5 mg. thiamine hydrochloride/l. Analar grade chemicals were used.

The medium was prepared in the following manner. The phosphates were mixed at ×4 final strength and adjusted to pH 6·5 with the appropriate phosphate solution. Sodium nitrate was added to the phosphate buffer at ×4 final strength. Glucose solutions were prepared at ×5 final strength and the EDTA chelated trace elements at ×20 final strength. Glucose solutions were autoclaved at 115° for 15 min., whilst the trace elements and phosphate+nitrate solutions were sterilized separately by autoclaving at 121° for 15 min.; 200 ml. of medium were prepared by mixing four solutions aseptically: 50 ml. of phosphate+nitrate, 40 ml. of glucose, 10 ml. of EDTA chelated trace elements and 100 ml. of water. For solid media 3 g. of Oxoid Ionagar no. 2 was added to the water (final concentration 15 g./l.).

When the effect of glucose concentration on growth was studied, the glassware used to prepare the medium was soaked in 2% quadralene (Fisons, Loughborough, Leicestershire) for 1 hr, washed six times in tap water and rinsed in distilled water.

Preparation of agar plates. 20 ml. volumes of medium were pipetted into 9 cm. sterile plastic Petri dishes which had been levelled on the tripod table described by Pirt (1967). The plates were dried at 37° for about 10 min. in the inverted position with the lids removed. The depth of medium in a plate was determined by measuring a thin vertical section cut from the medium with a razor blade.

Preparation of inocula and inoculation. Inocula were prepared by washing spores from McCartney slopes with about 10 ml. of 0·1 % Tween 80. The spores were washed twice with Ringer solution and then resuspended in sterile distilled water. Inocula used in spore germination experiments were kept up to 10 days at 4°, during which time there was no significant decrease in viability.

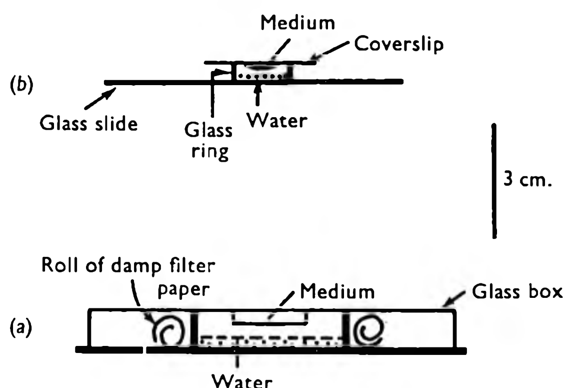

Fig. 1. Various culture techniques: (*a*) culture chamber used to follow growth microscopically, (*b*) slide culture used to follow growth microscopically.

Each plate was inoculated at three points with 1·5 to 2·0 μl. of spore suspension dispensed from a repette (Pirt, 1967). The plates were incubated in biscuit tins with beakers of water containing rolls of filter paper. Unless stated otherwise, *Aspergillus nidulans* was grown at 37° and *Penicillium chrysogenum* and *Mucor hiemalis* at 25°.

Measurements of spore and colony growth. Growth was recorded by measuring the diameter of colonies in two directions at right angles with a Shadowmaster (Pirt, 1967) and taking the mean of at least six replicates. Growth rates were calculated from measurements made at two intervals (9- to 18-hr period) whilst the colonies were growing at a constant rate.

The initial stages of colony growth and spore germination were followed by photographing the organism growing in a culture chamber or slide culture under a microscope (Fig. 1*a*, *b*). Photographs were taken at 15 min. intervals with a Shackman 35 mm. camera (Shackman & Sons, Chesham, Bucks.). Measurements were made on the projected film (final magnification ×650 or ×300). Experiments were done in constant-temperature rooms.

Measurement of hyphal density and internode length. The cultures were fixed with formal + acetic acid + ethanol (FAA) and stained with trypan blue in lactophenol (Righelato, Trinci, Pirt & Peat, 1968). The surface hyphae at the colony periphery were

photographed with a Zeiss photomicroscope. Hyphal density was determined by projecting the negatives (final magnification × 200) and counting the hyphae in focus crossing a 1000 μ arc 500 μ from the periphery of the colony. Each result was the mean of six replicates.

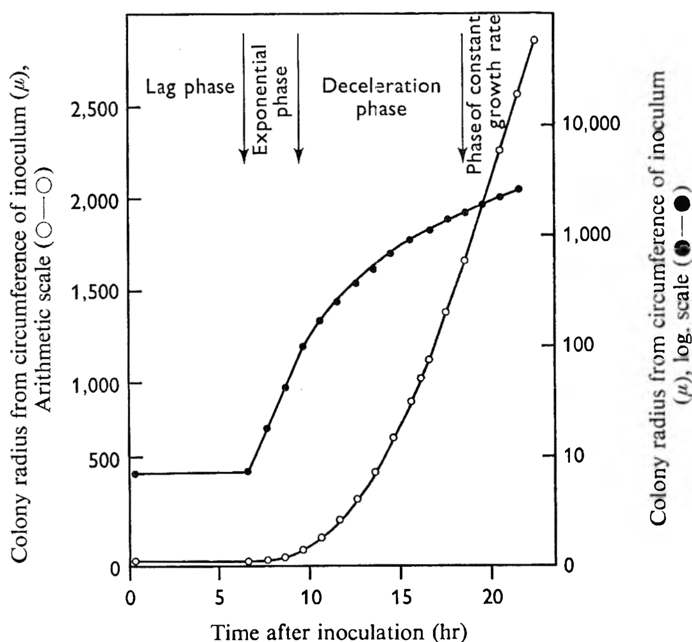

Fig. 2. Growth of surface colonies of *Aspergillus nidulans*.

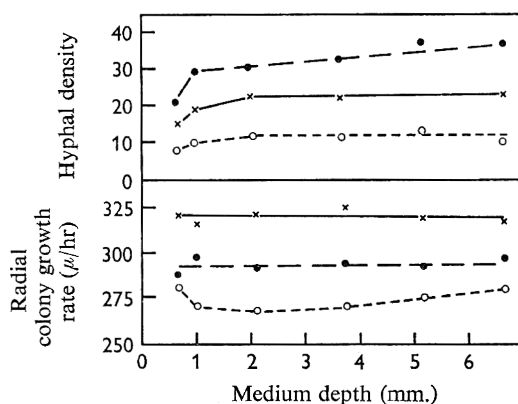

Fig. 3. Effect of medium depth on radial growth rate and hyphal density of *Aspergillus nidulans* colonies growing on 0·009 g. glucose/l. (○ --- ○), 0·16 g./l. (× —— ×) and 10·24 g./l. (● --- ●).

Internode length of *Mucor hiemalis* was the mean of the first six internodes of main leading hyphae (growth is monopodial). Hyphal diameter was measured 200 μ from the tips of main leading hyphae with a micrometer eyepiece. In both cases the results given are the mean of 6 hyphae per treatment.

Growth of colonies at various oxygen partial pressures. Cultures in vented Petri dishes were placed in anaerobic culture jars which were then flushed out with the oxygen + nitrogen gas mixtures (gas flow 1 l./min.) for 5 min. The anaerobic culture jars were lined with moist blotting paper and contained a beaker of 0·4% Pardee (1949) 'carbon dioxide buffer'.

Shake flask cultures. Submerged cultures were grown in 250 ml. conical flasks containing 25 ml. of medium. Each flask was inoculated with 1 ml. of spore suspension (final density 1·5 to $2·5 \times 10^6$ spores/ml. of medium) and incubated on a Gallenkamp rotary shaker (A. Gallenkamp & Co. Ltd., Christopher Street, London, E.C. 2) at 220 rev./min. Growth was followed by harvesting from flasks (at least 3/sample) with sintered glass crucibles (porosity X2). The mycelium was washed twice with 25 ml. of distilled water and dried overnight at 105°.

RESULTS

Phases of colony growth

Growth of *Aspergillus nidulans* colonies from the circumference of the inoculum was followed photographically (Fig. 2). Colony growth could be divided into four phases. After a lag phase the colony grew radially at an exponential rate (doubling time, 0·95 hr; specific growth rate, 0·73 hr^{-1}) until it was about 100 μ from the circumference of the inoculum. The radial growth rate of the colony continued to increase during the third phase but no longer exponentially. This deceleration (from the previous exponential rate) phase lasted until the colony had grown about 1·1 mm. from the inoculum circumference. During the final growth phase the colony grew at a constant radial rate; this is the so-called 'linear phase of growth'. Quite different kinetics would be obtained if the inoculum centre had been taken as the origin for growth measurements. The kinetics of colony growth are identical with those of the growth of unbranched hyphae (Trinci & Banbury, 1967).

Effect of medium depth on growth rate and hyphal density

Pirt (1967) found that medium depth had a significant influence on the growth rate of *Escherichia coli* colonies. To determine the effect of medium depth on the growth of *Aspergillus nidulans* colonies, plates were poured with different volumes of medium (5, 10, 15, 25, 35 and 45 ml., corresponding to depths of 0·66, 0·99, 2·10, 3·74, 5·18 and 6·68 mm. respectively). The organism was grown on three different glucose concentrations and the radial growth rate and hyphal densities of the colonies were determined (Fig. 3). Medium depth had no influence on colony growth rate but there was a significant increase in hyphal density with increase in medium depth on all but the lowest glucose concentration.

Colony growth into the medium

Filamentous micro-organisms are able to grow down into an agar medium as well as over its surface. To investigate the extent of the submerged part of *Aspergillus nidulans* colonies, a thin vertical section of an FAA-fixed colony was cut with a razor blade 6·5 mm. from its circumference. The section was stained with trypan blue in lactophenol, photographed along its length, and hyphal densities were determined at successive 0·5 mm. intervals (Fig. 4). The results showed that the rate of growth down

into the medium was apparently as fast as growth over the surface and that hyphal density decreased exponentially with depth below the surface of the colony. The colony was thus hemispherical in shape.

Effect of temperature on colony growth rate and germ tube specific growth rate on solid media and specific growth rate in submerged culture

Aspergillus nidulans was cultured at different temperatures to investigate the interrelationships between colony growth rate and germ-tube specific growth rate on solid media and specific growth rate in submerged culture.

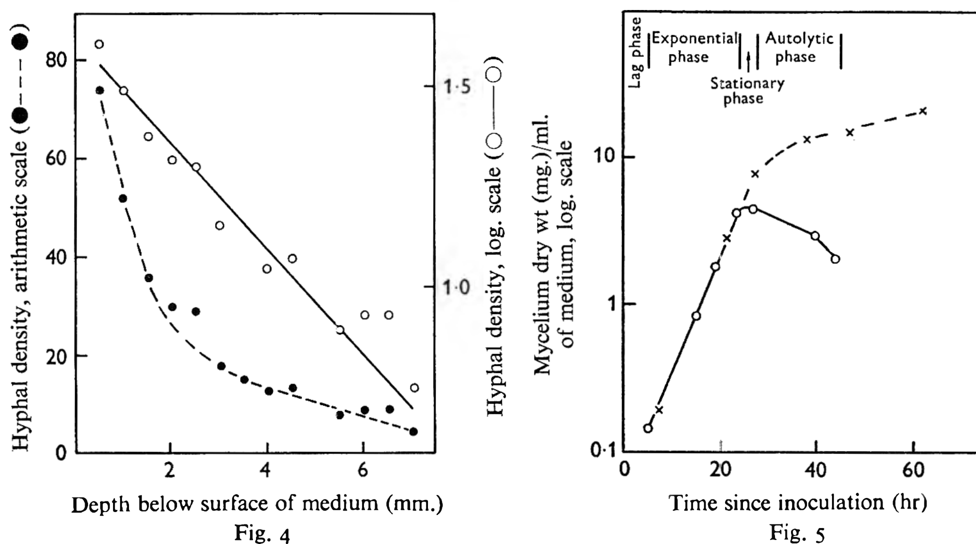

Fig. 4. Change in hyphal density down a vertical section cut 6·5 mm. from the circumference of an *Aspergillus nidulans* colony.

Fig. 5. Growth of *Aspergillus nidulans* in submerged culture on 10 g. glucose/l. (○——○) and 50 g./l. (× - - - ×).

Specific growth rate in submerged culture

Growth of *Aspergillus nidulans* in submerged culture at 30° is shown in Fig. 5. Growth of the organism in a medium containing 10 g. glucose/l. could be divided into lag, exponential, stationary and autolytic phases. Thus the growth kinetics of *A. nidulans* in submerged culture are similar to those commonly observed for unicellular micro-organisms. When *A. nidulans* was grown on a medium containing 50 g. glucose/l. there was a decrease in growth rate when the organism concentration exceeded 7 mg./ml. of culture (Fig. 5). This decrease may have been caused either by the accumulation of toxic products in the medium or by growth becoming limited by the availability of oxygen; the pH of the medium did not drop below 6·3. The concentration of carbon source in many mycological media exceeds 1 % (w/v) (it is 3 % in Czapek Dox and 4 % in Hesseltine medium for mucoraceous fungi), which, from these results, would seem to be too high for the maintenance of maximum specific growth rate in batch culture.

The specific growth rate of *Aspergillus nidulans* in submerged culture was determined over the temperature range 20° to 37° (Table 1).

Table 1. *Effect of temperature on colony radial growth rate and germ tube specific growth rate of Aspergillus nidulans on solid medium and specific growth rate in submerged culture*

Temp.	Colony radial growth rate (K_r, μ/hr)	Growth in submerged culture: Specific growth rate (α_s, hr^{-1})	Growth in submerged culture: Doubling time (t_{ds}, hr)	K_r/α_s	Germ tube growth: Specific growth rate (α_g, hr^{-1})	Germ tube growth: Doubling time (t_{dg}, hr)	K_r/α_g	α_g/α_s
20	86	0·090	7·72	958	0·193	3·60	446	2·14
25	146	0·148	4·68	985	0·371	1·90	394	2·50
30	215	0·215	3·23	1000	0·583	1·19	369	2·71
35	287	—	—	—	0·721	0·97	398	—
37	297	0·360*	1·96	835	0·730	0·95	407	2·03

The mean of the ratios were as follows: $K_r/\alpha_s = 942$, $K_r/\alpha_g = 403$ and $\alpha_g/\alpha_s = 2·35$. * Mean of four experiments.

Table 2. *Colony radial growth rate, germ tube specific growth rate and specific growth rate in submerged culture of Aspergillus nidulans, Penicillium chrysogenum and Mucor hiemalis at 25°*

Organism	Colony radial growth rate (K_r, μ/hr)	Growth in submerged culture: Specific growth rate (α_s, hr^{-1})	Growth in submerged culture: Doubling time (t_{ds}, hr)	K_r/α_s	Germ tube growth: Specific growth rate (α_g, hr^{-1})	Germ tube growth: Doubling time (t_{dg}, hr)	K_r/α_g	α_g/α_s
A. nidulans	146	0·148	4·68	986	0·371	1·90	394	2·50
P. chrysogenum	53	0·123	5·65	432	0·263	2·64	202	2·14
M. hiemalis	424	0·099	7·02	4296	0·587	1·18	722	5·93

Germ-tube specific growth rate

Conidial germination was followed photographically in slide cultures (Fig. 1 *c*) until the germ tubes were about 150 μ long. Growth of a germ tube at 37° is shown in Fig. 6; the diameter of the conidium was included in all measurements and when the spore formed a second germ tube its length was also included. Conidia grew exponentially from the time when the germ tubes emerged until they were about 120 μ long. Smith (1924) demonstrated the exponential nature of hyphal growth on solid medium but in his experiments measurements were only begun when the hyphae were about 230 μ long. Plomley (1959) reported that for the first few hours after germination the germ tubes of Chaetomium conidia grew at a constant rate, not exponentially.

The mean doubling times (t_{dg}) and specific growth rates (α_g, hr^{-1}) of germ tubes were calculated over the temperature range 20° to 37° (Table 1). When the conidia were not crowded together there was good agreement between the specific growth rates of germ tubes grown in different culture slides at the same temperature; e.g. at 37° the germ tube doubling times ranged from 53 to 62 min. with a mean of 57 min.

Colony radial growth rate

The growth rate of *Aspergillus nidulans* colonies at various temperatures was also determined (Table 1).

Inter-relationships

The results (Table 1) show that at least for part of the temperature range tested the changes in colony radial growth rate (K_r) reflected changes in specific growth rate in submerged culture (α_s, hr^{-1}). Between 20° and 30° the ratio, colony radial growth rate: specific growth rate in submerged culture (K_r/α_s) did not vary by more than 6·5% from the mean but at 37° the ratio was higher than at other temperatures and there was a 12·5% deviation from the mean. Pirt (1967), studying the growth of bacterial colonies, found a similar change in the ratio, colony growth rate: $\sqrt{}$(specific growth rate), at high temperatures. He interpreted his results as indicating the presence of a temperature sensitive coefficient in colony growth. Rather surprisingly it was found that the germ tube specific growth rate (α_g, hr^{-1}) was greater than the specific growth rate in submerged culture (α_s, hr^{-1}) by a factor varying from about 2 to 2·7 (Table 1). It was concluded that within experimental error the ratios, colony radial growth rate: specific growth rate in submerged culture (K_r/α_s), colony radial growth rate: germ tube specific growth rate (K_r/α_g) and germ tube specific growth rate: specific growth rate in submerged culture (α_g/α_s) were constants.

Colony growth rates and germ tube specific growth rates on solid media and specific growth rates in submerged culture of Aspergillus nidulans, Penicillium chrysogenum and Mucor hiemalis at 25°

The colony radial growth rates, germ tube specific growth rates and specific growth rates in submerged culture of *Aspergillus nidulans*, *Penicillium chrysogenum* and *Mucor hiemalis* at 25° were determined (Table 2). There was considerable variation between the three species in the ratio, colony radial growth rate: specific growth rate in submerged culture (K_r/α_s). *Mucor hiemalis* had the fastest colony growth rate but the slowest specific growth rate in submerged culture. Thus the growth rates of

colonies of the three species cannot be taken as reliable indication of their relative specific growth rates in submerged culture. The rapid growth of *M. hiemalis* on plates is sustained by the transport of protoplasm synthesized within the colony to the comparatively few leading hyphae at the colony's periphery. The absence of septa may enable a greater region of the colony to contribute to its radial extension than might be the case in septate species. Certainly rapid protoplasmic streaming in hyphae in the direction of the colony's circumference is commonly observed in mucoraceous fungi. Thus, although an organism may only be able to synthesize protoplasm at a comparatively low rate (i.e. it has a low specific growth rate) it may be able to sustain a rapid radial rate of colony growth.

Influence of glucose concentration on colony growth rate, hyphal density, hyphal diameter and internode length

Pirt (1967) found that the relation between glucose concentration in the medium when it was the growth limiting nutrient and the initial radial growth rate (K_r) of bacterial colonies could be represented by the formula:

$$K_r = k_1(\sqrt{S_0} - \sqrt{S_i}),$$

where k_1 was a constant, S_0 the initial glucose concentration and S_i the glucose concentration which had to be exceeded before growth of the colony could occur; S_i was termed the 'lag concentration' by Pirt. The following experiments were made to study the effect of glucose concentration on radial growth rate, hyphal density, hyphal diameter and internode length of fungal colonies.

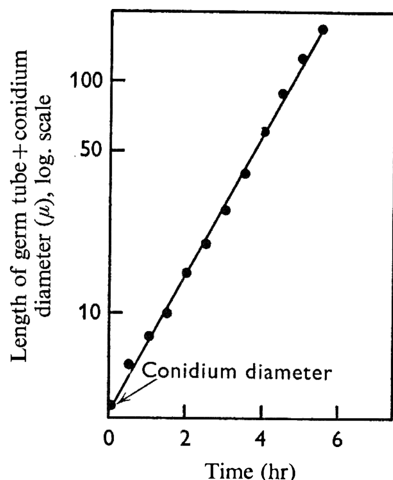

Fig. 6. Growth of an *Aspergillus nidulans* conidium.

The influence of glucose concentration on the growth of *Aspergillus nidulans*, *Mucor hiemalis* and *Penicillium chrysogenum* colonies was determined (Fig. 7 and 8). All three species grew rapidly even at the lowest glucose concentration (0·009 g./l.). At low glucose concentrations the growth of both *A. nidulans* and *M. hiemalis* colonies was glucose limited. The radial growth rate of *A. nidulans* colonies and the log. of the initial glucose concentration was linear over a considerable range (0·009 to 0·15 g./l.). The optimum glucose concentration for maximum colony growth rate was 0·075 g./l.

for *M. hiemalis* and 0·2 g./l. for *A. nidulans* (over the lower range). These concentrations are much lower than the minimum glucose concentration required for bacterial colonies to attain their maximum rate of growth (e.g. 2·4 g./l. for *Streptococcus faecalis* and 4·0 g./l. for *Escherichia coli*; Pirt, 1967). At glucose concentrations greater than the optimum there was a decrease in the growth rate of *A. nidulans* and *M. hiemalis* colonies; in both species the colony radial growth rate declined linearly with the log. of the initial glucose concentration. At glucose concentrations above 10·24 g./l. there was a second acceleration phase in the rate of growth of *A. nidulans* colonies.

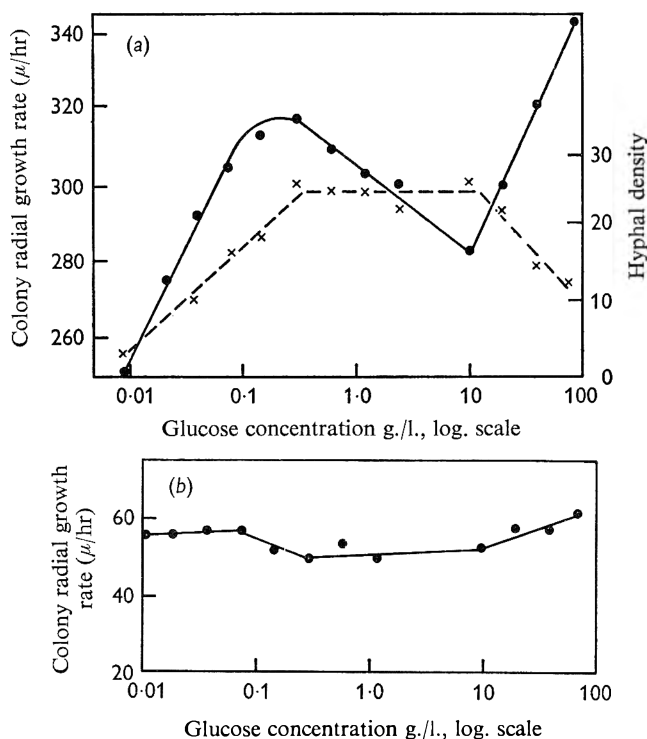

Fig. 7. (*a*) Effect of glucose concentration on the radial growth rate (●——●) and hyphal density (× - - - ×) of *Aspergillus nidulans* colonies. (*b*) Effect of glucose concentration on the radial growth rate of *Penicillium chrysogenum* colonies.

The peripheral hyphal density of *Aspergillus nidulans* colonies increased linearly with the log. of the initial glucose concentration between 0·009 and 0·3 g./l. and the decline in growth rate at higher concentrations was correlated with the attainment of the maximum hyphal density. The acceleration in growth rate of *A. nidulans* colonies which commenced at 20·48 g./l. was correlated with a decline in hyphal density; there was an inverse relationship between colony radial growth rate and hyphal density.

The mean internode length of the main leading hyphae (Fig. 8*a*) of *Mucor hiemalis* colonies was not influenced by glucose concentration between 0·19 and 81·92 g./l. but there was a significant decrease in internode length at 0·009 g./l. Internode length is meaningless in *A. nidulans* since the hyphae of this organism branch apically as well as laterally (A. P. J. Trinci, unpublished observation). There was an increase in the mean diameter of the main leading hyphae of *M. hiemalis* colonies between 0·009 and

1·2 g. glucose/l. (Fig. 8*b*). At glucose concentrations above 1·2 g./l. hyphal diameter was apparently constant. The mean total length of the first six branches of *M. hiemalis* was also determined (Fig. 8*b*).

Growth of colonies of the slow-growing organism, *Penicillium chrysogenum*, was not glucose limited even at 0·009 g./l.; growth of this organism must be limited by some other factor.

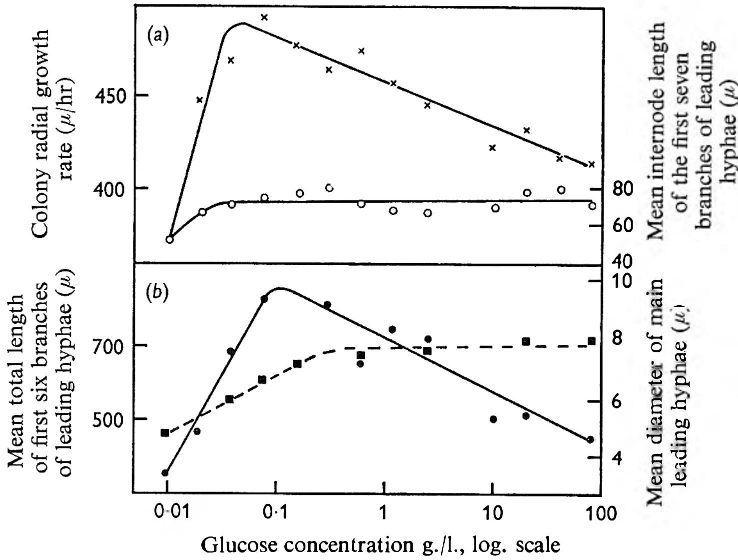

Fig. 8. (*a*) Effect of glucose concentration on the radial growth rate (×——×) and hyphal internode length (○——○) of *Mucor hiemalis* colonies. (*b*) Effect of glucose concentration on the total length of the first six branches of leading hyphae (●——●) and the diameter of leading hyphae (■ - - - ■) of *Mucor hiemalis* colonies.

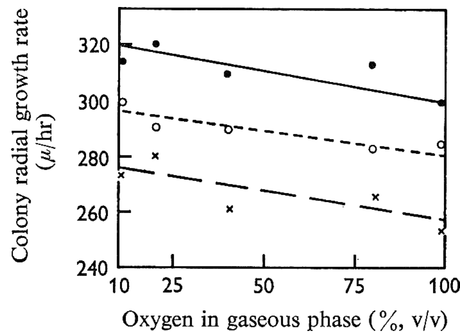

Fig. 9. Effect of oxygen concentration on the radial growth rate of *Aspergillus nidulans* colonies grown on 0·1 g. glucose/l. (○ - - - ○), 10 g./l. (× - - - ×) and 81·92 g./l. (●——●)

Effect of glucose concentration on germ tube specific growth rate

The specific growth rate (mean of 3 to 4 replicates) of *Aspergillus nidulans* germ tubes growing on media containing different glucose concentrations in culture chambers (Fig. 1*a*) was determined (Table 3). There was little variation in the germ tube specific growth rate over the glucose range tested. Germ tube growth was probably

not limited by external glucose concentration because the conidia had sufficient endogenous supplies of energy-containing compounds for germination. Thus germ tube specific growth rate probably cannot be used as a parameter of growth when studying the effect of nutrient concentration. However, the experiment demonstrates that high external glucose concentrations were not inhibitory to growth.

Influence of oxygen partial pressure on colony growth rate

Pirt (1967) found that at glucose concentrations of 2·5 g./l. and above the radial rate of growth of *Escherichia coli* colonies was limited by oxygen availability. The inhibition of growth of *Aspergillus nidulans* colonies at glucose concentrations between 0·2 and 10·24 g./l. (Fig. 7*a*) may have been caused by oxygen becoming the growth limiting factor.

Table 3. *Influence of glucose concentration on the doubling time and specific growth rate of germ tubes of Aspergillus nidulans*

Glucose concentration g./l. of medium	Mean doubling time (t_{dg}, hr)	Mean specific growth rate (α_g, hr^{-1})
0·009	1·30	0·5331*
0·038	1·10	0·6300
0·150	1·15	0·6026
0·300	1·10	0·6300
1·200	0·98	0·7071
10·240	1·15	0·6026
40·960	1·12	0·6187
81·920	1·18	0·5873

* The germ tube specific growth rate was slightly lower in the culture chambers than in the culture slides (Fig. 1).

Aspergillus nidulans colonies were grown in air for 24 hr, measured and then grown for a further 18 hr at different oxygen partial pressures. At each glucose concentration growth was fastest at an oxygen concentration of 10%, v/v and slowest at 100% (Fig. 9); thus the growth rate of *A. nidulans* colonies is not oxygen limited even when grown on 81·92 g. glucose/l.

DISCUSSION

Growth of colonies of filamentous and unicellular micro-organisms differ in several respects. Initiation of the growth of bacterial colonies occurs only when the glucose concentration in the medium exceeds a certain value called the 'lag concentration' (Pirt, 1967); the lag concentration for *Escherichia coli*, *Klebsiella aerogenes* and *Streptococcus faecalis* was 0·090, 0·013 and 0·005 g. glucose/l. respectively. However, all three fungal species in the present study grew rapidly on media containing 0·009 g. glucose/l. In addition fungal colonies attain their maximum radial growth rate at a significantly lower glucose concentration than bacterial colonies (e.g. 0·075 g./l. for *Mucor hiemalis* as compared to 4·0 g./l. for *E. coli*). The filamentous habit permits variation in colony density such that the energy source available in the medium is used to maximum efficiency in extending the colony's diameter. The hyphal density control mechanism is presumably of significant selective advantage as it enables moulds to

spread across substrates containing low concentrations of nutrients at near maximum radial growth rates; when a substrate containing a high concentration of nutrients is encountered, the colony grows more densely, produces aerial hyphae and may sporulate. Unicellular micro-organisms do not possess a specific mechanism to control the density of growth within the colony and typically form small colonies. A further advantage imparted by the filamentous habit is that it facilitates penetration of solid media and as a result the organism obtains nutrients which usually only become available to unicellular micro-organisms by diffusion. The radial growth rate of *Aspergillus nidulans* colonies, unlike *Escherichia coli* colonies, (Pirt, 1967) was never found to be limited by oxygen availability. Fungal colonies never attain the density of bacterial and yeast colonies at their periphery and it is probably because of this that the growth of leading hyphase does not become oxygen limited.

Internode length of *Mucor hiemalis* main hyphae was not influenced by glucose concentration between 0·0018 and 8·192% (w/v). Lateral branch initiation is thus apparently not significantly influenced by nutrient concentration; differences in hyphal density result from variation in the number of branches which continue to grow once formed rather than by variation in the frequency of branch formation along main hyphae.

Colony radial growth rate increases linearly with the log. of the initial glucose concentration when this is the growth limiting nutrient. Gillie (1968) found that the rate of extension of *Neurospora crassa* colonies was proportional to the log. of the arginine concentration over the lower range, and it would thus seem that this is a general relationship for any growth limiting nutrient.

Synthesis of protoplasm contributing to radial extension of a colony is not confined to the tips of leading hyphae; Zalokar (1959*b*) showed that in *Neurospora crassa* portions of hyphae as far removed as 12 mm. from the periphery of a colony contributed to the growth of its leading hyphae. If the environmental conditions within this peripheral growth zone became unfavourable to growth, the radial growth rate of the colony would decrease. The finding that germ tube specific growth rate of *Aspergillus nidulans* was not influenced by glucose concentration is an indication that high glucose concentrations are not in themselves inhibitory to growth. Thus the reduction in radial growth rate of *A. nidulans* and *Mucor hiemalis* colonies when growth was not glucose limited is probably due to changes in the medium resulting from growth of the organism, i.e. the accumulation of toxic products or pH changes. The rate at which these changes occur would presumably be related to hyphal density. The rapid growth of *A. nidulans* colonies on media containing 81·92 g. glucose/l. was associated with a substantial decrease in hyphal density. The radial growth rates of *A. nidulans* colonies on media containing 1 and 5% glucose (w/v) were different (Fig. 7*a*), although the mould had the same maximum specific growth rate on these media in submerged culture (Fig. 5). Thus colony radial growth rate is not a reliable parameter of specific growth rate in submerged culture in studies where nutrient concentration is varied.

Changes in specific growth rate of *A. nidulans* in submerged culture brought about by temperature changes were reflected in colony radial growth rate at least over most of the range tested. Colony radial growth rate would thus seem to be a reliable parameter to use to determine the optimum temperature for growth of a mould. However, it is clear from the results obtained that colony radial growth rate is not a meaningful

parameter of growth (i.e. of specific growth rate in submerged culture) when comparisons are made between species.

Germ tube specific growth rate may prove to be a useful parameter of growth to use in certain bioassays and physiological investigations in which the effect on growth of factors other than nutrient concentration are being studied. The technique is less time consuming and more economical of media than submerged culture.

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Action of Hydrogen Peroxide on Growth Inhibition of *Salmonella typhimurium*

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SUMMARY

The effect of hydrogen peroxide on the growth of *Salmonella typhimurium* LT2 in a mineral glucose medium was investigated. The H_2O_2 produced a lag period, the duration of which increased as the concentration of H_2O_2 in the medium was increased from 1 to 60 μg./ml. Growth subsequent to the lag period proceeded at the normal growth rate at concentrations of H_2O_2 as high as 30 μg./ml. Storage of H_2O_2 in the sterile growth medium resulted in a disappearance of H_2O_2 with a half-life of about 48 hr. The disappearance of H_2O_2 because of reaction with glucose resulted in proportionate decreases in the growth inhibitory action of the medium. *Salmonella typhimurium* destroyed H_2O_2 rapidly (half-time = 60 min.)—an effect attributed largely to bacterial catalase. The catalatic activity of the bacteria was decreased or eliminated by boiling or by treatment with cyanide. The growth of the bacteria in H_2O_2 resulted in the development of H_2O_2 resistance. By subculturing the wild type LT2 in medium containing successively higher H_2O_2 concentration, a resistant strain designated LT2P was isolated which grew in the presence of H_2O_2 concentrations that were completely inhibitory to the wild type.

The experimental results support the generally accepted conclusion that bacterial catalase acts in protecting the organism from H_2O_2. It is pointed out that in biological media H_2O_2 readily forms adducts with many compounds, including carbonyls, amino acids and thymine. Consequently, the inhibitory effects of H_2O_2, especially in studies involving auxotrophs, may be partly or wholly due to the peroxide adduct rather than 'free' H_2O_2.

INTRODUCTION

In studies on the biological effects of irradiated media, we utilized the growth response of *Salmonella typhimurium* strain LT2 as one measure for the detection of radiolytically generated toxic products (Schubert, Watson & White, 1967; Schubert & Watson, 1969; Schubert, Watson, & Baecker, 1969). Since hydrogen peroxide (H_2O_2) is one of the primary molecular products produced by the action of ionizing radiation in aqueous solutions (Baxendale, 1964), we conducted a series of experiments to define the effect of chemically added H_2O_2 in our microbiological assay test system.

Most microbiological investigations have utilized survival (colony forming ability) for evaluating the toxic effects of H_2O_2. One of the few investigations dealing with the inhibitory effect of H_2O_2 on the exponential phase of growth was reported by Campbell & Dimmick (1966). These investigators, using *Serratia marcescens*, compared survival with growth after exposure to very high concentrations of H_2O_2 (30 mg./ml.).

Survival was not affected until after about 4 min. of contact with the solution, whereas the growth response changed immediately after contact. The latter changes were manifested over 40 hr by changes in the duration of the lag phase, in the growth rate and in maximal growth.

Frey & Pollard (1966) demonstrated that considerable periods of lag followed by normal growth resulted when exponentially growing bacteria of an *Escherichia coli* autotroph were inoculated into irradiated media. The duration of the lag period was dependent on the amount of H_2O_2 produced in the medium by radiolysis. The inhibitory action of the irradiated medium or of chemically added H_2O_2 could be eliminated by addition of catalase. The susceptibility of bacteria to H_2O_2 generally correlates with the catalase content of the organisms (McLeod & Gordon, 1923; Molland, 1947; Amin & Olson, 1968). However, no consistent correlation is obtained between sensitivity to ionizing radiation and catalase content (Engel & Adler, 1961; Adler & Clayton, 1962; Adler, 1963).

METHODS

Bacterial strain. Salmonella typhimurium strain LT2 was obtained from Professor E. Englesberg. The culture was maintained on nutrient agar (Difco) slants enriched with nutrient broth (Difco) to give an 0·8 % (w/v) solution.

Media. Bacteria were grown in a mineral glucose medium (pH 7·0) of the following percentage (w/v) composition: KH_2PO_4, 0·3; K_2HPO_4, 0·7; $(NH_4)_2SO_4$, 0·1; $MgSO_4 . 7H_2O$, 0·1 and glucose, 0·05, as sole source of carbon and energy (Englesberg, 1959). The carbon source and salts were sterilized separately by Millipore filtration and added as concentrated solutions. Desired concentrations of H_2O_2 were prepared by adding appropriate volumes of freshly prepared solutions of reagent grade H_2O_2 (non-stabilized) in distilled water to the above medium immediately before the addition of the bacteria.

Growth measurements. Glucose-grown organisms, harvested during the exponential phase of growth by centrifugation at 3° and washed once in saline, were inoculated into 18 × 150 mm. growth tubes containing the desired defined medium (final volume, 5 ml.) at a concentration of $5·7 \times 10^7$ bacteria/ml. (Englesberg, 1959). The tubes were incubated at 37° on a rotary shaker and the turbidity was measured at designated intervals using a Fisher electrophotometer (427 mμ filter; blank, sterile medium) modified to accept the growth tubes (Englesberg, 1959). Growth measurements, based upon the average of duplicate tubes, were recorded in Fisher units (1 Fisher unit = $1·14 \times 10^7$ bacteria/ml. = 5·8 μg. dry weight of bacteria/ml.). The logarithms to the base 2 of the Fisher units have been presented to facilitate the comparisons of growth rates.

Hydrogen peroxide determination. Hydrogen peroxide was measured by the titanium sulphate method (Egerton *et al.* 1954). Analyses were made after the removal of the organisms by centrifugation for 10 min. at 10,000 *g* at 3°.

Catalase. Stock solutions of catalase were prepared before use from beef liver catalase powder (Sigma Chemical Company, Inc., St. Louis, Missouri). Three μg./ml. of the catalase preparation in mineral glucose medium decomposed 24·5 μg. H_2O_2/ml. in 15 min. at pH 7·0 and 37° (see below).

RESULTS

Effect of H_2O_2 on the growth of Salmonella typhimurium. The effect of various concentrations of H_2O_2 on the growth of the bacteria in mineral glucose (0·05 %) medium is shown in Fig. 1. The exponential growth rate in the absence of H_2O_2 was 1·18 divisions per hr. The duration of the lag period increased as the H_2O_2 concentration was increased to 60 µg./ml. The growth rate subsequent to the lag period showed little change up to an H_2O_2 concentration of 30 µg./ml. By 22 hr the same degree of maximal growth on glucose was reached at all H_2O_2 concentrations tested.

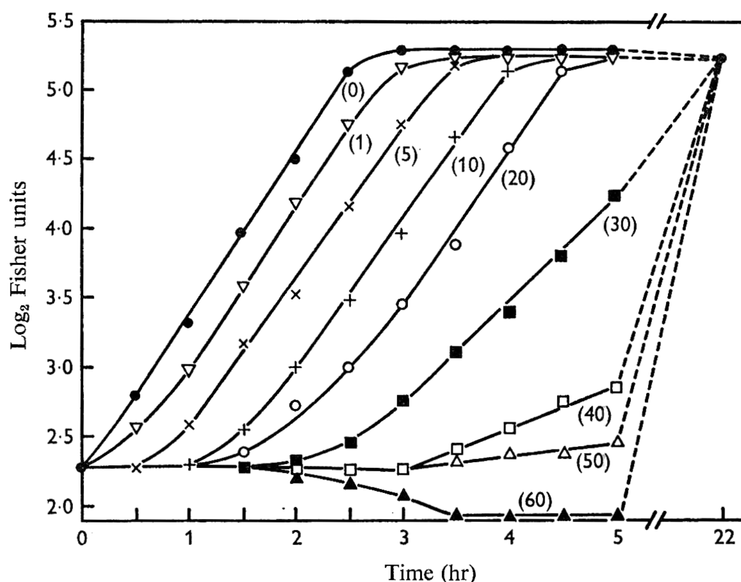


Fig. 1. Effect of increasing concentrations of hydrogen peroxide on the growth of *S. typhimurium* in a mineral glucose (0·05 %) medium. Numbers in parentheses represent the µg. H_2O_2/ml. added to the medium prior to inoculation with the cells. Inoculum: $5·7 \times 10^7$ organisms/ml.

Effect of storage time on the inhibitory action of hydrogen peroxide. We tested the effect of dark storage at 37° in sterile mineral glucose medium (pH 7·0) on the inhibitory action of H_2O_2. Figure 2 summarizes the results obtained when growth measurements were performed daily over a 5-day period with stored medium whose initial H_2O_2 concentration was 60 µg./ml. (These results are also representative of those obtained at lower peroxide concentrations.) A progressive loss in the inhibitory action of the stored solution occurred and after 5 days the growth response of the inoculated bacteria was indistinguishable from that of controls grown in the absence of H_2O_2. No bacterial contamination occurred in the stored media.

Moody (1963) showed that H_2O_2 reacts with glucose during incubation at low temperatures. The observed loss in inhibitory action of H_2O_2 in our stored medium corresponded to the decrease in H_2O_2 levels resulting from an interaction with glucose. Figure 3 shows representative results obtained in stored media with an initial H_2O_2 concentration of 20 µg./ml. Little change in the H_2O_2 concentration occurred over a 165-hr period in the stored growth medium in which glucose was omitted, while the

H_2O_2 disappeared with a half-life of approximately 48 hr in the medium containing glucose. These results are in agreement with our earlier findings (Schubert *et al.* 1969).

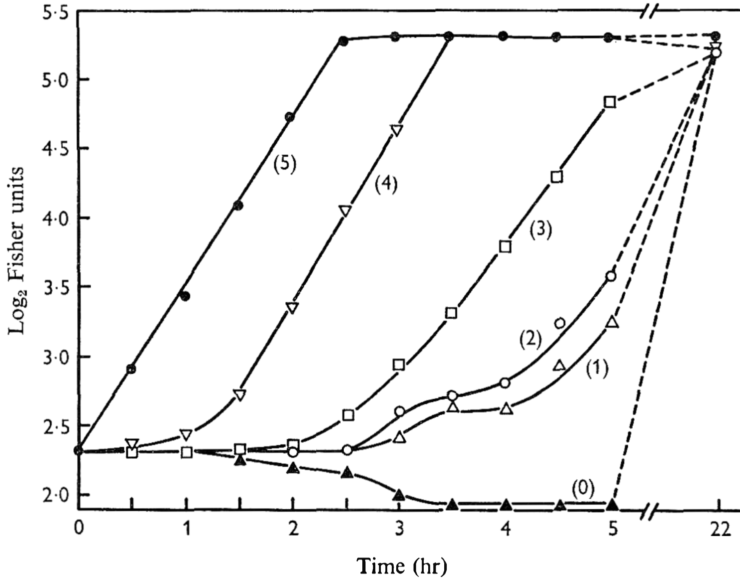
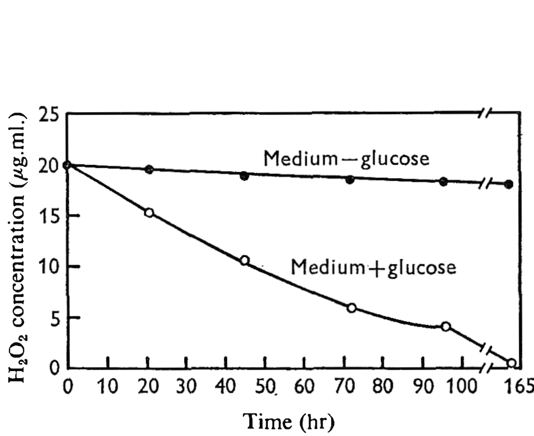


Fig. 2. Effect of storage time at 37° in mineral glucose (0·05 %) medium (pH 7·0) on the inhibitory action of hydrogen peroxide. Initial H_2O_2 concentration (0): 60 μg./ml. Numbers in parentheses represent the days of storage. Inoculum: $5 \cdot 7 \times 10^7$ organisms/ml.



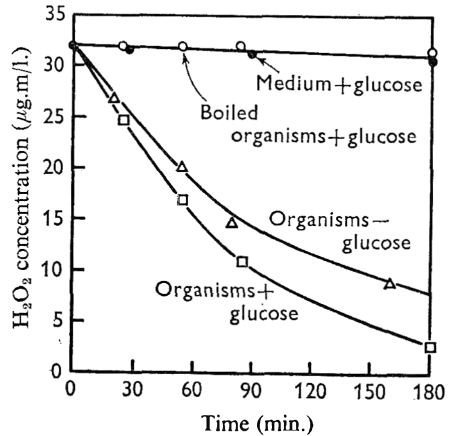


Fig. 3. Effect of glucose (0·05 %) on the disappearance of hydrogen peroxide from growth medium (pH 7·0) at 37°. Initial H_2O_2 concentration: 20 μg./ml. Hydrogen peroxide was measured by the titanium sulphate method.

Fig. 4. Effect of *S. typhimurium* in the presence and absence of glucose on hydrogen peroxide disappearance. H_2O_2 concentration: 32 μg./ml. Inactivated organisms were boiled for 15 min. in distilled water and were resuspended in saline to a turbidity equivalent to $5 \cdot 7 \times 10^7$ bacteria/ml. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the organisms by centrifugation.

Destruction of hydrogen peroxide by Salmonella typhimurium. The effect of *S. typhimurium* on the destruction of H_2O_2 was investigated. The bacteria were incubated in mineral medium containing 32 μg. H_2O_2/ml. in the presence and absence of glucose. Bacteria inactivated by boiling for 15 min. were similarly treated in glucose-containing medium. Hydrogen peroxide disappearance was measured at designated times over a 3-hr period (Fig. 4).

The growth response of the bacteria in the medium containing glucose was similar to that shown in Fig. 1 for the 30 μg. H_2O_2/ml. level in which the lag phase persisted for about 2 hr before exponential growth resumed. The data shown in Fig. 4 therefore describes the rate of destruction of H_2O_2 during the lag phase. The half-time of H_2O_2 disappearance was about 1 hr. At the point where the lag phase ended about 80% of the H_2O_2 originally in the medium had disappeared. H_2O_2 destruction by the organisms in the absence of glucose was appreciable but occurred at a lower rate than that obtained with the 'growing' organisms in glucose-containing medium. The disappearance of H_2O_2 from glucose medium in the presence of organisms inactivated by boiling did not differ significantly from the rate of loss obtained over 3 hr in the mineral glucose medium alone. We assume, as have most investigators, that the catalatic activity of the bacteria is largely due to catalase. We have not attempted to measure the catalase activity following degradation of the organisms (Weibull & Hammarberg, 1963; Frey & Pollard, 1966) because we believe that the catalatic activity of the intact viable organism is more meaningful and less likely to lead to contradictory results.

The effect on H_2O_2 disappearance following chemical inhibition of catalase was tested with potassium cyanide (KCN), a known catalase inhibitor (Nicholls & Schonbaum, 1963). Increasing amounts of KCN were added to sterile growth medium and the destruction of H_2O_2 by 3 μg. purified beef liver catalase/ml. ($1{\cdot}2 \times 10^{-8}$M)

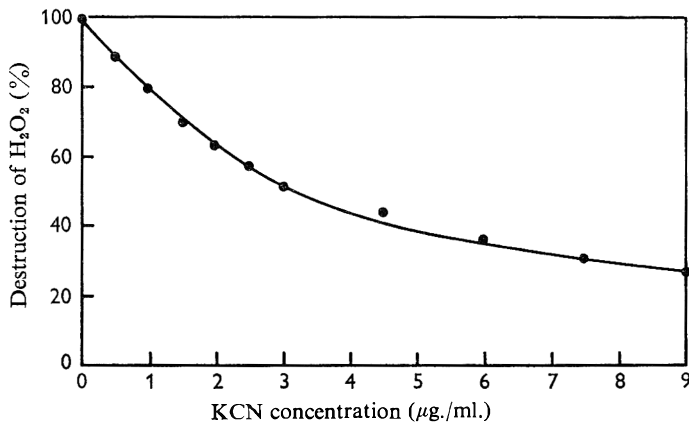


Fig. 5. Effect of potassium cyanide on the destruction of hydrogen peroxide by catalase. Four ml. volumes of complete mineral glucose medium (pH 7·0) containing 3 μg. catalase/ml. in the presence and absence of various concentrations of KCN were incubated for 15 min. at 37°. Two ml. of H_2O_2 containing medium were then added to each to give a total volume of 6 ml. The medium was incubated for 15 min. at 37° and was assayed for H_2O_2 by the titanium sulphate method. Initial H_2O_2 concentration: 32 μg./ml. Under the above conditions, 24·5 μg. were destroyed by 3 μg. catalase/ml. in the absence of KCN. Values plotted below are expressed as a percentage of the latter value.

was measured. The destruction of H_2O_2 decreased as the KCN content of the system increased (Fig. 5). However, even in the presence of a large molar excess (approximately 10^4) of KCN, complete inactivation of the catalase was not obtained under the conditions of this test.

The growth and H_2O_2 disappearance of KCN-treated organisms in the absence of glucose is shown in Fig. 6. Growth in mineral glucose medium containing 20 μg. KCN/ml. was inhibited for at least 4 hr (Fig. 6A) but by 22 hr reached approximately one half the level of maximal growth attained by the glucose and H_2O_2 (34 μg./ml.) controls. A drop in the turbidity of the suspension treated with KCN + H_2O_2 in the absence of glucose occurred during a 4-hr period (Fig. 6A). Hydrogen peroxide disappearance by KCN-treated organisms in the absence of glucose was markedly decreased (Fig. 6B). These results are compatible with an inactivation of the bacterial catalase by the inhibitor.

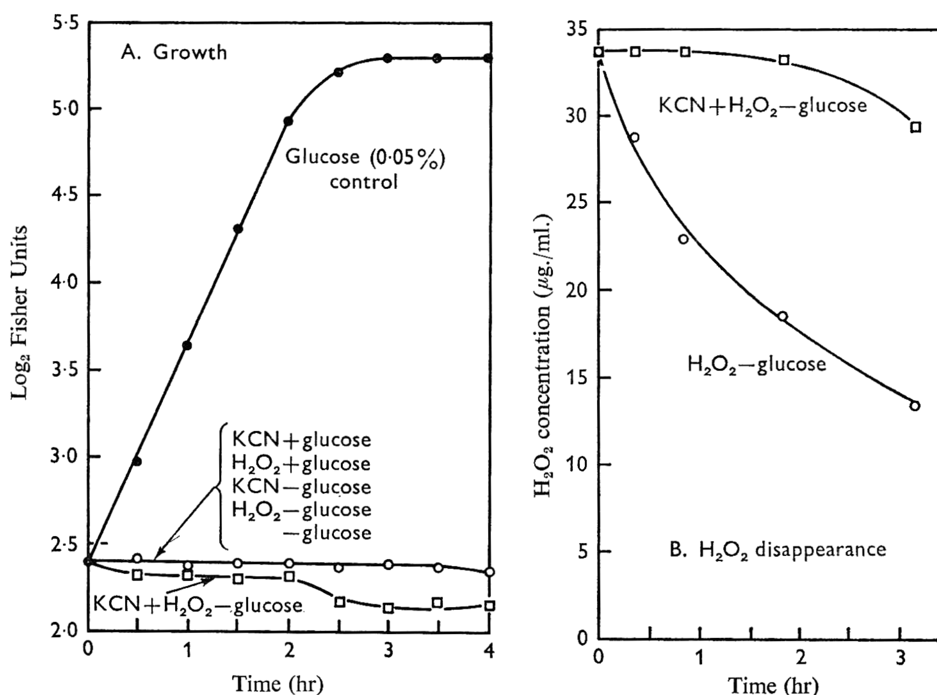


Fig. 6. Effect of a 15 min. pretreatment (37°) with potassium cyanide (20 μg./ml.) on growth (A) and hydrogen peroxide disappearance (B) by *S. typhimurium* in mineral medium in the *absence* of glucose. Initial H_2O_2 concentration: 34 μg./ml. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the cells by centrifugation. Inoculum: $5{\cdot}7 \times 10^7$ organisms/ml.

Development of hydrogen peroxide resistance. The effect of a previous exposure to H_2O_2 on the ability of the organisms to respond to a subsequent H_2O_2 exposure was tested. Figure 7 shows the growth response obtained when 20 μg. H_2O_2/ml. were added to organisms growing in the presence and absence of 20 μg. H_2O_2/ml. The H_2O_2 was added when the cell-mass had doubled in the respective media. The response of the organisms growing previously in the absence of H_2O_2 was characterized by a

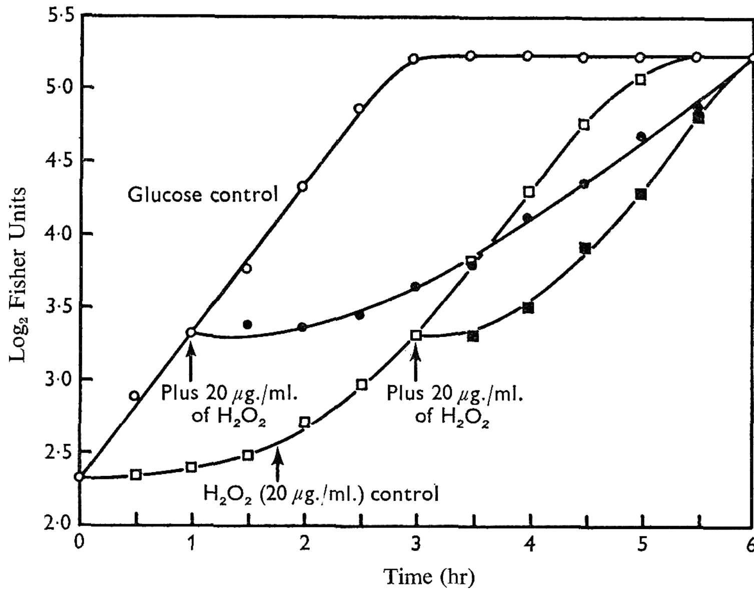


Fig. 7. Effect of adding 20 μg. hydrogen peroxide/ml. on the growth of *S. typhimurium* in mineral glucose (0·05 %) medium and in mineral glucose medium containing 20 μg./ml. of hydrogen peroxide. The H_2O_2 was added to each growth tube in a total volume of 0·2 ml. when growth had doubled in the respective media. Two tenths (0·2) ml. of distilled water was added to the control tubes at the same time. Inoculum: $5 \cdot 7 \times 10^7$ organisms/ml.

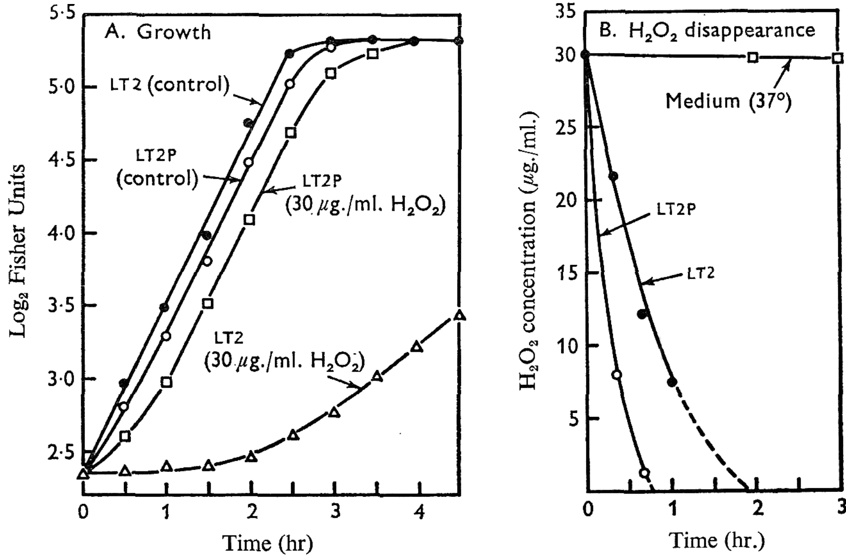


Fig. 8. Growth in mineral glucose (0·05 %) medium in the presence and absence of 30 μg. H_2O_2/ml. (A) and hydrogen peroxide disappearance (B) by the wild type (LT2) and peroxide resistant (LT2P) strains. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the cells by centrifugation. Inoculum: $5 \cdot 7 \times 10^7$ organisms/ml.

more prolonged lag phase and by an inhibition in the growth rate, as compared to the response of the organisms previously exposed to H_2O_2. The results suggest that the concentration of catalase in the organisms was raised following growth in the presence of H_2O_2.

To determine if growth in the presence of H_2O_2 resulted in the selection of an H_2O_2 resistant strain, wild type LT2 were sub-cultured daily into mineral glucose medium containing successively higher H_2O_2 concentrations. Following twelve such transfers, a strain achieving maximal growth within 22 hr in mineral glucose medium containing 600 μg. H_2O_2/ml. was isolated. This strain, designated LT2P, retained the ability to grow in the presence of H_2O_2 concentrations that were completely inhibitory to the wild type following eight successive transfers in the absence of H_2O_2. Figure 8 shows the growth (Fig. 8A) and H_2O_2 disappearance (Fig. 8B) of the LT2 and LT2P strains in the presence of an H_2O_2 concentration (30 μg./ml.) permitting the growth of the wild type. The growth rate of the LT2P strain in the absence of H_2O_2 was slightly lower than the normal LT2 response and only a slight inhibition in growth is produced by 30 μg. H_2O_2/ml. (Fig. 8A). The resistant strain removed H_2O_2 at a rate about 3·5 times greater than did the wild type (Fig. 8B).

DISCUSSION

The inhibitory action of H_2O_2 on the growth of *Salmonella typhimurium* manifests itself primarily in the production of a lag phase, the duration of which is proportional to the concentration of H_2O_2. No effect on the maximal growth reached at 22 hr in the glucose medium occurred at H_2O_2 concentrations to 60 μg./ml., nor were significant changes observed in the growth rate subsequent to the lag until 30 μg. H_2O_2/ml. was reached (Fig. 1). These results, obtained with an inoculum consisting of $5{\cdot}7 \times 10^7$ exponential phase bacteria/ml. are qualitatively similar to the response shown by *Serratia marcescens* (Campbell & Dimmick, 1966) and *Escherichia coli* (Frey & Pollard, 1966) to H_2O_2 under different experimental conditions.

Our results support the generally accepted conclusion that the catalatic activity of the organism plays an important role in protection against H_2O_2 (Fig. 4, 6). In studies reported elsewhere (Schubert & Watson, 1969), it was shown that the addition of catalase to H_2O_2 containing medium before inoculation with *Salmonella typhimurium* eliminated the lag phase completely. The addition of catalase at increasing times after inoculation, however, had a decreasing influence on the duration of the lag produced at the H_2O_2 concentration tested. When H_2O_2 was in contact with the organisms for about 45 min. before catalase was added, no effect on the subsequent duration of the lag phase was observed. Figure 4 shows that approximately 60% of the initial H_2O_2 in the medium was still available at this time. The mechanism by which H_2O_2 exerts this effect on the growth capability of the bacterial cell has been attributed to a type of repairable injury which affects cell division (Campbell & Dimmick, 1966).

The H_2O_2-resistant strain of *Salmonella typhimurium* isolated following repeated contact with H_2O_2 (Fig. 8) is of potential usefulness for evaluating the presence of toxic products other than H_2O_2 in irradiated media containing high H_2O_2 levels. Although we have isolated a number of strains which show an apparent increase in resistance to H_2O_2 when haemin is added to the growth meduim (Beljanski, 1955), we

have adopted the use of the former strain for this purpose since no supplementation of mineral glucose medium is required. In previous work we have shown that H_2O_2 interacts with organic molecules present in the growth medium. The loss of toxicity of stored growth medium containing H_2O_2 (Fig. 2), resulting from a glucose/H_2O_2 interaction (Fig. 3), is one example of this effect. Of greater significance, however, is the fact that H_2O_2 has been shown to react with carbonyl compounds, e.g. glyoxal, glycolaldehyde (Schubert *et al.* 1967) and histidine (Schubert *et al.* 1969) to form adducts which are more toxic to the growth of *S. typhimurium* than the individual compounds alone. These adducts may be readily decomposed by catalase (Weitzel, Buddecke & Schneider, 1961; Schubert *et al.* 1969). Similar increases in the toxic and mutagenic properties of various organic molecules following treatment with H_2O_2 have been shown in other biological systems (Wyss, Stone & Clark, 1947; Weitzel *et al.* 1961). In the report by Frey & Pollard (1966) a thymine-requiring auxotroph was used to study the effect of added H_2O_2. In unpublished work we have found that thymine reacts with H_2O_2 to form an adduct which is more toxic than H_2O_2 itself. We would suggest, therefore, that the use of auxotrophs as test systems for evaluating the biological effects of H_2O_2 or of irradiated solutions should be supplemented by studies with the corresponding wild-type strains.

The results of the present study are relevant to the effects produced by irradiated solutions on this microbiological test system. It should be emphasized, however, that we do not feel that H_2O_2 itself is the primary cause of biological damage, since it is highly unlikely that H_2O_2 ever exists free in a biological milieu (J. Schubert, to be published). It is our opinion that H_2O_2 adducts formed by interaction with various degradation products produced during irradiation are responsible for the antibacterial properties of irradiated media.

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Dispersal of Streptomycetes in Air

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SUMMARY

The number of streptomycete propagules trapped from air above a fallow plot depended on the amount of dust in the air. Less than 20% of the propagules occurred as individuals; the remainder were mostly attached to the surface of airborne soil particles. Thus any action which disturbed the soil surface and launched soil particles into the air, such as the operation of an agricultural implement or a gusty wind, also increased the concentration of airborne streptomycete propagules.

With simulated rain, the first water drops which struck the soil surface launched both individual spores and spores attached to soil particles into the air; with time the water drops washed most propagules from the air.

INTRODUCTION

Streptomycetes are widely distributed in soils, especially in those which are dry and not too acid and those rich in organic matter; here, in propagule numbers, they frequently exceed the combined counts of all other bacteria (Alexander, 1961; Waksman, 1959). Spread of streptomycetes in soil is by active filamentous growth or by passive movement of spores by soil animals, water or gravity.

Streptomycete spores may also be dispersed aerially. So far, most studies on aerial dispersion have been concerned with the aetiology of certain respiratory diseases associated with inhaling spores of actinomycetes, for example, farmer's lung disease (Gregory & Lacey, 1963). Here, millions of actinomycete spores per g. of hay were liberated into the air when mouldy hay was shaken. Less is known of the movement of streptomycetes that occur in soil, and the purpose of this study was to examine aerial dissemination of streptomycete propagules originating from soil. The work was also of interest to the South Australian Asthma Foundation, which supplied the Andersen sampler.

METHODS

Air was sampled above a plot of land formerly used as a nursery for peach trees, then cropped once with beans and finally left fallow for a year. This plot, approximately 40 m. by 21 m., was bordered on one side by an orchard and on the other sides by other cultivated plots, all within the grounds of the Waite Agricultural Research Institute, South Australia.

The soil (Urrbrae loam) is a red-brown earth with a silty loam texture and pronounced fine sand fraction (Piper, 1938). Soil from this plot, when stored, contained approximately $2 \cdot 0 \times 10^6$ streptomycetes and $11 \cdot 5 \times 10^6$ other bacteria per g. dry weight

as determined on dilution-plates of chitin agar (Lingappa & Lockwood, 1962; Lloyd, Noveroske & Lockwood, 1965) for estimating streptomycetes, and soil agar (Bunt & Rovira, 1955) for bacteria.

Airborne propagules of streptomycetes were collected above the plot with an Andersen air sampler (Andersen, 1958), placed so that its base was 45 cm. above ground level and near the centre of the field. The operator remained stationary during the time of air sampling to avoid unnecessary disturbance to the soil surface. Petri dishes within the sampler contained chitin agar, sometimes supplemented with acitidione (cycloheximide) at 20 μg./ml., although frequently this was not required, as chitin agar is selective for streptomycetes. After drawing 0·14 m.^{3} of air through the sampler, the Petri dishes were incubated at 28° for 6 to 8 days. Colonies of streptomycete growing on the chitin agar were then counted.

A sensitive-cup anemometer placed adjacent to the Andersen sampler and with the cups 85 cm. above ground level measured windspeed during each sampling period, while rainfall was recorded by a gauge some 450 m. distant.

RESULTS

Concentration of propagules in air

Air was sampled above the fallow plot at 14.00 hr on 30 separate days between 13 December 1966 and 17 February 1967, and at hourly intervals on several selected days. The average number of streptomycete propagules trapped was 157 per m.^{3} of

Table 1. *Increase in concentration of airborne propagules of streptomycetes after disturbing the soil sufficiently to contaminate the air with dust*

Date	Soil treatment	Wind speed (m./sec.)	Airborne propagules (m.^{3} air)
4 Jan.	Undisturbed soil	1·4	129
5 Jan.	Dust raised by a cultivator operating in an adjacent field some 30 m. windward of air sampler	1·4	1043
26 Jan.	Undisturbed soil	2·5	36
26 Jan.	Soil lightly raked 8 m. windward of air sampler	3·3	643
1 Feb.	Undisturbed soil	1·5	14
	Soil lightly raked 8 m. windward of air sampler	1·0	364
	As above but in gusty wind	2·1	1857
14 Feb.	Undisturbed soil	1·2	0
	Cultivator operating on windward side of air sampler	1·2	1270
	Cultivator operating on leeward side of air sampler	1·2	764
	Cultivated soil lightly raked on windward side of air sampler	1·7	6715
	3 hr later without further disturbance of soil	1·7	200
	9 hr later without further disturbance of soil	1·0	98

air, while the highest number trapped at any one sampling time was 1,043 per m.^{3} of air; the lowest was 0 on one day only (Fig. 1). Concentration of propagules in air sampled once a day at 14.00 hr did not seem directly related to either windspeed as measured during air sampling (Fig. 1) or the daily rainfall, although, as shown later, simulated rain had a marked effect. Nor was the concentration of propagules consistently higher at any one time of day, except in the evening, when the number often increased with the onset of the local 'gully wind' characteristic of that area.

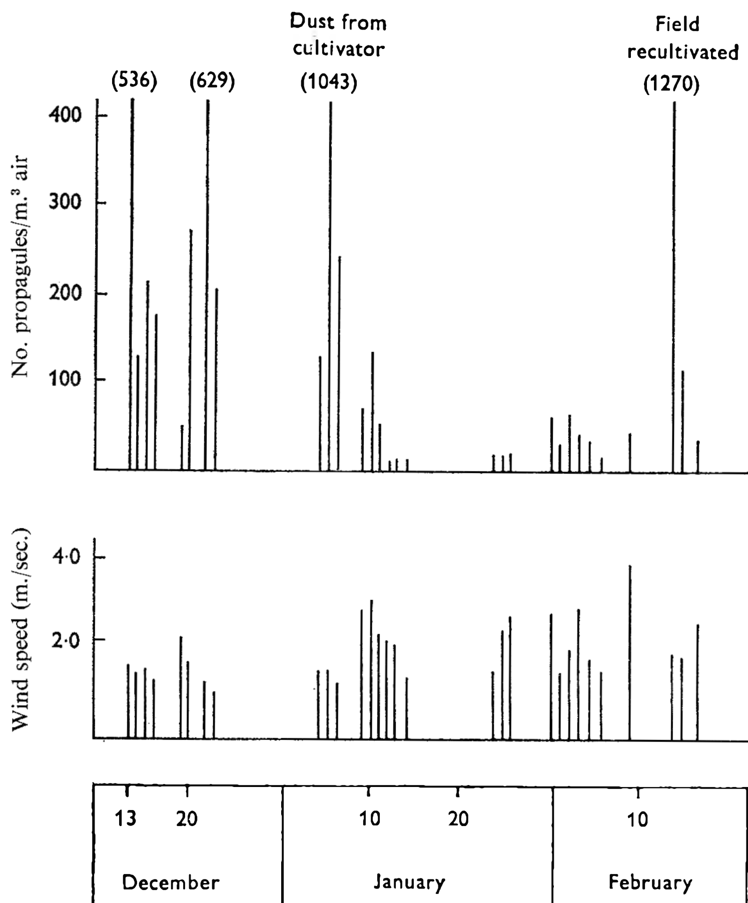

Fig. 1. Numbers of streptomycete propagules trapped from air above a fallow field on 30 separate days. Mean windspeeds during sampling periods are given as histograms below the propagule counts. Gaps are periods when air was not sampled.

Whenever dust contaminated the air, concentration of airborne propagules increased. Thus on 5 January a tractor-drawn cultivator, operating in an adjacent orchard, raised dust which drifted across the sampling site and propagule counts in the air reached the peak of 1043/m.^{3} (Fig. 1, Table 1).

Several other small experiments demonstrated that whenever soil was disturbed sufficiently to raise dust the number of airborne propagules of streptomycetes increased also (Table 1). Thus when the surface of soil 8 m. to the windward of the air sampler

was lightly raked, the concentration of airborne propagules increased from 14 to 364/m.³. Then by chance the wind became gusty and propagule concentration increased to 1857/m.³. Later the same fallow field was cultivated and when the freshly cultivated soil was lightly raked on the windward side of the air sampler, airborne propagules of streptomycetes increased from 0 to the high value of 6715/m.³. Three hours later, without further disturbance of the cultivated soil, concentration of airborne propagules was 200/m.³ and after 9 hr, 98/m.³.

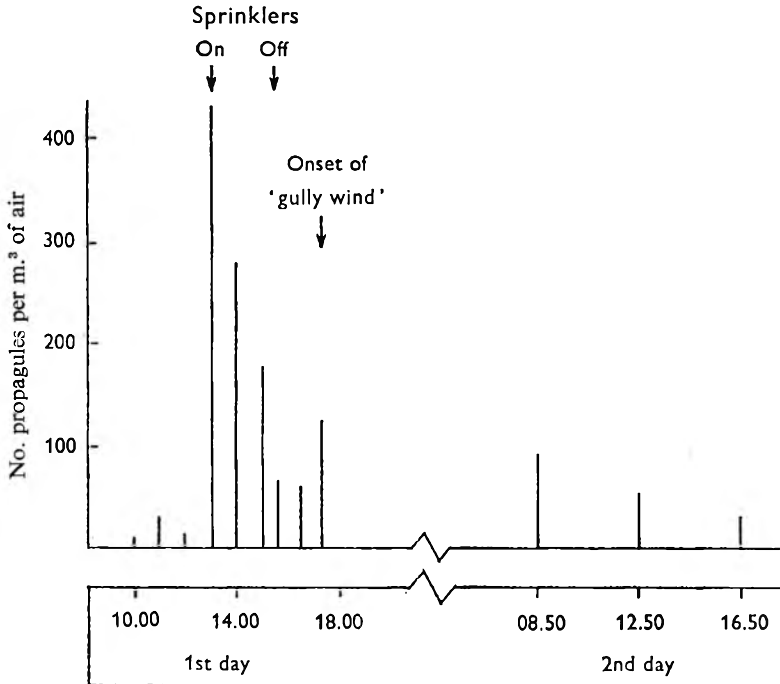

Fig. 2. Numbers of streptomycete propagules trapped from air above a fallow field before, during and after sprinkler irrigation. Gaps are periods when air was not sampled.

Effect of simulated rain on concentration of airborne propagules

The period between mid-December and mid-February is part of the dry season in South Australia: 33 mm. rain fell during that period in 1966–67. Therefore, to simulate rain, five sprinklers were used to apply water drops to the soil surface, and air was sampled at hourly intervals.

Concentration of streptomycete propagules in air above the sampling site just prior to watering was 20/m.³ of air (Fig. 2). This increased to 436/m.³ immediately after turning on the sprinklers. Thereafter, the concentration of airborne propagules decreased steadily throughout the period of simulated rain until it reached 71/m.³ when the water was turned off, some 2½ hr later (Fig. 2). For the remaining part of the afternoon the number of propagules remained low until the onset of the local 'gully wind' in the evening, when propagule numbers again increased. The following day was extremely hot, the soil surface dried out rapidly and a crust formed; concurrently the concentration of airborne propagules decreased until it reached 28/m.³ in the late afternoon.

Adherence of airborne spores to dust particles

The Andersen sampler is so designed that it separates particles of similar density into separate size ranges as they are collected on the surface of agar in a series of six Petri dishes (Andersen, 1958).

From all the air samples collected above the fallow field, 44% of the trapped propagules of streptomycetes appeared in stage 1 of the sampler (> 9 μ particle size), whereas only 17% appeared in stages 5 and 6 combined (< 2 μ). Further, if only the the air samples collected above undisturbed soil are considered, then 55% of all propagules appeared in stage 1 of the air sampler, and only 19% in stages 5 and 6 combined.

If a streptomycete spore is assumed to be spherical, of unit density and with diameter < 2 μ, then less than 20% of all streptomycete propagules collected from air occurred as individual spores, i.e. those trapped in stages 5 and 6, whereas some 80% were either attached to dust particles or occurred in spore clumps. It is more likely that the former is true, for when agar dishes were taken from stage 1 of the sampler and examined with a microscope, there was frequently a small grain of soil at the centre of each developing streptomycete colony. No such grains appeared in stages 5 and 6. Thus, it seems that most streptomycetes were aerially transported on fine soil particles, and relatively few as individual spores.

DISCUSSION

In air above a fallow plot most airborne propagules of streptomycetes were present not as individual units, but adhering to the surface of fine soil particles. Gregory & Lacey (1963) shook hay in a wind-tunnel and then captured actinomycete propagules, sampling the air at a rate similar to mine. Some 50% of all actinomycete propagules trapped from air were as individual spores, hence when actinomycetes grow on hay and then sporulate, the spores are liberated into the air mainly as individuals.

Aerial dispersal of streptomycete spores from soil is different, for within the soil streptomycetes grow vegetatively for short periods, sporulate and then the vegetative mycelium disappears, leaving behind localized regions of high spore concentration (Lloyd, 1969). The spores may become dispersed by animal movement and such passive means as water movement and gravity; but they frequently adhere to the surface of soil particles. It is in this attached state that most streptomycete spores are aerially dispersed. Thus any action which raises soil particles from the soil surface will therefore launch into the air streptomycete spores absorbed to the dust particles.

Simulated rain initially increases the concentration of streptomycete propagules in air. Apparently the first water drops, striking the soil surface, launch both individual spores and spores adhering to soil particles into the air. But with time the water drops wash propagules from the air.

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Light and Electron Microscopy of the Sheath of a Blue-green Alga

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SUMMARY

Light microscopy observations of a *Nostoc* sp. of blue-green alga suggested that the sheath was comparable with the capsules of many bacteria. The sheath showed little internal differentiation under normal or phase-contrast illumination. It possessed a somewhat hazy outer margin from which portions sloughed off, it stained with Alcian blue and was probably polysaccharide. The sheath formed salt-like complexes with proteins at appropriate low pH values which rendered it visible by phase-contrast microscopy. The composition of the culture medium influenced sheath formation. Electron microscopy showed that the sheath consisted of a micro-fibrillar network containing small numbers of larger fibrils (about 200 Å in diameter) which was compressed into stria near to the cell wall and expanded into an open reticulum further out. It would appear to be formed by continuous secretion through the longitudinal walls of the trichomes, and as the outermost regions imbibed water the network structure expanded and finally sloughed off.

INTRODUCTION

One of the most conspicuous characters of the blue-green algae (Myoxphyceae, Cyanophyceae) is the production of sheath substance of mucilaginous nature which is reminiscent of the capsules and mucoid substances produced by bacteria (Echlin & Morris, 1965). In the latter group these materials generally form homogenous structureless envelopes around the organisms by which they are secreted, although occasionally they possess a quite complex morphology (e.g. Labaw & Mosley, 1954; Tomcsik & Guex-Holzer, 1954). Among the blue-green algae there is a much greater variety of form and structure (see, for example, the review by Fritsch, 1945). The isolation of a heavily sheathed blue-green alga in this laboratory prompted an examination of this structure by using a variety of light and electron microscopy techniques, with a view to describing its nature and possible function in the ecology of this and other blue-green algae.

METHODS

Isolation and identification of organism. The algal isolate (FP 23) was obtained from a green film of algal growth found lining a plastic plant pot when it was emptied. The organism was isolated (May 1966) from crude cultures prepared in liquid media A and B (see below). It was alternately cultured in solid and liquid versions of medium A and appeared to be a uni-algal, but not axenic, culture at the time of these observa-

tions. Subsequently single-colony isolates have been picked to render it undoubtedly uni-algal. It is regarded as a *Nostoc* sp. as it conforms to the descriptions of this genus given by Fritsch (1945) and Smith (1950).

Cultivation. Cultures were maintained in Knop medium (Pringsheim, 1949) and the medium given by Stanier, Doudoroff & Adelberg (1963, table 19-6) without a nitrogen source (referred to as medium A) or with NH_4Cl (medium B). Bulk cultures were prepared in 1 l. volumes in half-gallon wine bottles which were slowly rotated (8 rev./min.) about 12 to 15 in. (30 to 38 cm.) below 4×40 W white fluorescent tubes. Paddles affixed to the walls of the bottles agitated the medium, which was periodically gassed with a 7 to 10% (v/v) CO_2-in-air mixture. Smaller quantities (25 ml.) were grown in 100 ml. Erlenmeyer flasks under similar illumination. All incubation was at room temperature (26° to 28° in the medium) for 7 to 14 days. Cultures were routinely handled aseptically in sterile containers although freshly prepared Knop medium for the bulk cultures was not always sterilized. No differences were observed in such cultures from those made in sterilized media. For plate cultures, 1·5% (w/v) agar was added to the liquid media.

Light microscopy. A Leitz Labolux microscope with phase-contrast optics was used routinely. Photomicrographs were made with a Leitz Ortholux microscope and Orthomat camera on Kodak Plus X film. All preparations were made without special fixation or drying to avoid artifacts due to distortion of organisms and sheaths. McKinney's (1953) capsule stain was used as described by her, but was subsequently replaced by mounting filaments directly in aqueous Alcian blue (0·5 to 1·0% w/v). Indian ink mounts were made with methods and precautions described by Duguid (1951). Non-specific protein staining of the sheath was achieved by Tomcsik & Guex-Holzer's (1954) method adapted as described below.

Electron microscopy. Fixation was by the Kellenberger–Ryter standard method (described by Glauert, 1965), and embedding was in Araldite. Some material was prefixed in glutaraldehyde and subsequently with osmium tetroxide (Glauert & Thornley, 1966). Sections were cut at a nominal 500 Å thickness (grey/silver colour) with an LKB Ultratome I ultramicrotome and mounted on carbon reinforced collodion membranes on copper grids. After staining with Karnofsky's lead hydroxide (Glauert, 1965) the preparations were examined in a JEM model T6 microscope, operating at 60 kV. Photomicrographs taken on Ilford NS50 plates at initial magnifications of $\times 5,000$ to $\times 12,000$ were printed at a further $\times 3$ to $\times 8$ magnification.

RESULTS

Growth

In the roll cultures (medium A) growth occurred as finely granular suspended dark-green clumps and attached to the walls as much lighter isolated pale-green jelly-like colonies, or as a nodular gelatinous sheet. The proportion of wall to suspended growth varied from bottle to bottle, even within batches put up at the same time. Cultures showing markedly gelatinous growth forms were studied in detail. Little or no sheath formation occurred in medium B.

Indian ink preparations

Teased portions of growth mounted carefully in Indian ink to avoid undue compression of the filaments possessed wide well-defined gelatinous sheaths around almost all trichomes (i.e. the chains of cells inside the sheaths), whether the cells were healthy or in various stages of degeneration (Pl. 1, fig. 1*a*, *b*). Sheaths averaged 18 μ wide (range 10 to 29 μ). Healthy cells had a diameter of about 4 μ. Degenerate cells were distorted and shrunken, some to mere specks. The outer limits of the sheath substance were not distinct and strands of sheath substance could be seen free and separating from the sheath surface around degenerate or lysed cells. Diffuse 'slime' layers were not seen.

Larger clumps of algal growth had the appearance of a completely transparent jelly in which were embedded long sinuous trichomes. In older cultures, the trichomes were often coiled in a loose spiral fashion within the sheath which itself retained a straighter course (Pl. 1, fig. 2). Possibly this spiralling within the sheath presaged degeneration because spiral coils of dead cells within wide sheaths were very common in old cultures. The sheaths were of uniform width except where heterocysts and akinetes had formed; at the former sites they narrowed to half size or less. Filaments frequently ruptured at heterocyst sites and the sheath rounded off at this point, leaving half the terminal heterocyst outside the sheath (Pl. 1, fig. 1*a*). The sheath also appeared to degenerate around akinetes in old filaments where it sloughed, became irregular in breadth, and partly infiltrated by the ink particles; ultimately it released individual akinetes.

Phase-contrast microscopy

The sheath was almost invisible under normal illumination, though its presence was sometimes betrayed by attached bacteria. Under phase-contrast microscopy visibility was generally no better, although the bacteria naturally became more distinct. Sometimes the features seen clearly with Alcian blue and protein could just be made out.

Alcian blue-stained preparations

Alcian blue is a stain widely used in histopathology as a specific stain for polysaccharides, and its use for the demonstration of bacterial (polysaccharide) capsules was developed by McKinney (1953). Her method worked with the alga FP23, but sheath stainability was weak, and phase microscopy of material mounted in dilute Alcian blue (about 0·5 to 1 % w/v) was more satisfactory. Most stain was taken up at the sheath surface, the bulk of the sheath substance being only faintly stained. Internal striae which appeared to be condensations of intensely stained material could also be seen (Pl. 1, fig. 4). In some areas these striae appeared to split, expand and became less intensely stained. There were rarely more than three layers. Cross-striations in line with the cross-walls of underlying cells were frequently seen.

Filaments differed greatly in the extent to which they stained. Narrow sheaths and sheaths around old trichomes tended to stain most intensely (sometimes very deeply), while sheaths around apparently young vigorously growing trichomes took up very little stain. Staining was usually poor around akinetes and weaker or even absent in the narrow sheath zones next to heterocysts. Cell walls and undamaged cells were unstained. Broken cells stained readily.

Non-specific protein absorption by sheath

Tomcsik & Guex-Holzer (1954) showed the ability of capsules of various bacteria to 'stain' or absorb a variety of proteins and give a non-specific 'capsule swelling' reaction which made them visible under phase-contrast microscopy. It was thought that blue-green algal sheaths might react similarly. Growth from a two week culture of alga 1P23 in Knop medium was washed twice with normal saline, resuspended in normal saline to give about 4·5 mg. dry weight/ml. and mixed with an equal volume of 0·1 % v/v bovine serum albumen (BSA) or about 3 % w/v sheep haemoglobin in distilled water. Volumes (0·5 ml.) of this mixture were then mixed with equal volumes of McIlvaine buffer and samples were examined mounted wet under coverslips by phase-contrast microscopy. Control preparations, examined in parallel with test preparations, consisted of algal growth treated exactly as above, but without added protein.

Optimum sheath visibility occurred at about pH 3·4 with both proteins, but values on either side of this showed protein uptake by the sheath. The sheaths in BSA-treated preparations, at pH 3·4, when washed with buffer at pH 7·2 became virtually invisible under phase-contrast microscopy, and this process could be followed microscopically with suitably mounted preparations (Pl. 1, fig. 3*a*, *b*). Examination of preparations at pH 3·4 in the presence of Indian ink to confirm the coincidence of the phase-contrast visible sheath and ink-outlined sheath resulted in coagulation of the ink. Observations with nigrosin resulted in some negative staining, but (unexpectedly) also in a marked positive staining of the protein-treated sheath. These observations will be recorded elsewhere.

The microscopic picture of the protein stained or protein + nigrosin stained sheaths was exactly comparable with that seen in Alcian blue preparations and (very faintly) in phase-contrast microscopy. Stain uptake was concentrated at the sheath surface and on longitudinal striations which tended to split, broaden and become less intensely stained. The sheath at heterocyst and akinete sites showed changes similar to those described above. The haemoglobin-treated sheaths were very intensely stained at values below pH 4·6 (note the much higher concentration of protein used) and sometimes gave the impression even of shrinkage.

Electron microscopy

The sheath substance was well preserved by the fixation techniques used, but intracellular details were sometimes poorly visible, especially when the sheath was thick (presumably fixative penetration was hindered). The internal structure of the cells will not be described here; but photosynthetic lamellae, α and β granules, ribosomes and nuclear material, and a wall structure (Pl. 2, fig. 6) now regarded as typical of the blue-green algae (Echlin & Morris, 1965) were observed.

Except that the outer investment and the innermost layers of the sheath seemed to be distinct structures, it has not been possible to describe the immediate cell-wall/sheath relationships in detail. This was partly because of lack of resolution, but mainly because these two structures became separated during processing, probably during dehydration. Variations in the dehydration procedure or solutions have so far not overcome this problem.

Cultures grown in medium B formed either a small thin almost electron-transparent sheath, or no sheath at all. In both osmium tetroxide and glutaraldehyde + osmium

tetroxide fixed material from cultures in Knop medium the sheath was readily seen in ultrathin sections. In young vigorously growing cultures it showed a dense striate structure next to the cells (Pl. 2, fig. 7; Pl. 3, fig. 9). Progressively further out from the cells the striae became looser and more open, they split lengthwise, expanded and became less densely stained. In the outermost regions the sheath structure comprised a fine micro-fibrillar network in which the meshes were elongated along the filament. The finest fibrils were estimated to have a diameter of about 50 to 70 Å. The number and density of striations close to the cell walls, and the amount of expansion in the outer layers, varied enormously from one filament to another. Sometimes the sheath was represented by numerous tightly packed dense layers with little or no fraying at the edges, and sometimes there were only one or two distinct layers and a much wider zone of the expanded network. The outer zones of adjacent filaments merged imperceptibly; sometimes sloughing of the outer zones was obvious. Filaments cut in cross-section showed the sheath as a ring of more or less tightly packed striations, expanding into a micro-fibrillar network at the periphery. Oblique sections showed that the lamellations themselves comprised aggregates of close-packed micro-fibrils, most of them oriented more or less parallel to the filament axis (Pl. 3, fig. 8). The extreme edges of these sheaths were probably never seen in sections, because the stainable electron-opaque material at this point was too sparse.

In the circumferential valleys formed at cell junctions (Pl. 2, fig. 7; Pl. 4, fig. 13), the sheath texture differed. Layering was absent, and this space was filled with even-meshed network of very fine (or sometimes less fine) micro-fibrils, reflecting differences in degrees of packing or staining. The unlayered appearance can be interpreted by considering the valley to be filled with small amounts of newly secreted sheath substance which expanded to fill the space created beneath older pre-existing sheath layers as the cells began to pull apart. Its open mesh would therefore be of the type found in the outermost unrestricted sheath layers.

Filaments from old cultures showing signs of degeneration lost their striate sheath structure and the sheath became a uniform homogenous mass of electron-transparent material. At the edges, the usual micro-fibrillar network could be seen, merging with the surrounding medium (Pl. 4, fig. 11). Sometimes empty sheaths were found whose cores were filled with a faint network.

The bacteria present in the cultures were very evident in sections; they were never found immediately adjacent to the algal cell walls in sheathed filaments, but they were sometimes found in the middle sheath layers, and they were very often found in the outer layers (Pl. 4, fig. 11). Large numbers occurred in the vicinity of degenerate cells and trichomes, smaller numbers around healthy trichomes. They were capable of colonizing empty sheaths, and small micro-colonies (presumably clones) were observed enclosed within a portion of some sheaths. The bacterial types involved could not be identified in the sections, though at least two morphological forms could be distinguished—small regular rods about $0{\cdot}25 \times 0{\cdot}5\ \mu$, and larger more pleomorphic forms about $0{\cdot}5\ \mu$ diam. The smaller forms were sometimes deeply embedded in the sheath, but the larger forms were restricted to the outermost zones. Sometimes the texture of the sheath next to the embedded bacteria seemed different from portions remote from bacteria, but consistent changes were not noted. Bacteria were frequently found embedded in sheath material right in the centre of masses of algal growth and in the disintegrating sheaths around akinetes.

The sheath substance generally collapsed in metal-shadowed preparations, and only a faint 'halo', marked by the change in texture of the background, could be seen. At high magnification this appeared to be network of 40 to 70 Å diameter micro-fibrils in suitably oriented areas. In a few filaments the collapsed sheath contained numerous more or less parallel fibrils from 170 to 350 Å in diameter, mostly lying along the length of the filament (Pl. 5, fig. 14 to 16). Anastomoses and splitting of these macro-fibrils could be seen. Anastomoses made fibril length somewhat indeterminate, but some fibrils could be traced for 1·5 to 3 μ along the sheath; those running across reached about 0·8 μ in length. Between and overlying these macro-fibrils could be seen areas of the more usual micro-fibrillar network. The macro-fibrillar zone was comparatively narrow—even dried and flattened its width was only 2·5 to 4·5 μ, compared with the 17 μ measured in living Indian ink preparations, or the 6·5 to 10 μ of the narrowest sheaths measured by phase-contrast microscopy. Beyond this well-defined zone with sharp edges could be seen the faint electron-transparent zone described above. It has so far not been possible to identify these macro-fibrils in sections.

DISCUSSION

The mucilaginous structure described in this paper is generally referred to as a 'sheath' in standard works on the Myxophyceae (e.g. Smith, 1950; Fritsch, 1945; Desikachary, 1959). Is this structure comparable with sheath and capsular structures observed (less commonly) among the other procaryotic micro-organisms the bacteria? Bacterial sheaths and capsules are usually differentiated morphologically; the former is a hollow structure surrounding a trichome or chain of cells, where the latter is a more closely applied envelope with or without a well defined margin (e.g. Skerman, 1967). Empty sheaths, but not empty capsules, are frequently noted. Romano & Peloquin (1963) showed that the sheath of the bacterium *Sphaerotilus natans* is chemically distinct from the capsule; the former comprises a protein–polysaccharide–lipid complex and the latter a polysaccharide only.

The ready stainability with Alcian blue suggests that the algal sheath is largely polysaccharide (McKinney, 1953), and reported chemical analysis on other blue-green algae makes this likely. Polysaccharides of various compositions have been isolated from species of the genera *Rivularia*, *Calothrix*, *Nostoc* and *Anabaena* (O'Colla, 1962). Moore & Tischer (1964) found the 'capsule' polysaccharide (their terminology) of a *Nostoc* sp. to consist of arabinose, glucuronic acid and fucose, and in *Anabaena flos-aquae*, largely of glucose and xylose with minor amounts of glucuronic acid and ribose (Moore & Tischer, 1965). In this latter paper these authors also showed that the sheath polysaccharides were likely to be synthesized intracellularly, and then to diffuse out of the cell through the wall. If the rate of accumulation at the cell surface were to exceed the rate of absorption of water in the more superficial layers of the sheath, one might expect to find deeper layers more electron-dense than the outer layers. As more water was absorbed the outermost layers would slough and go into solution. (The amount of water taken up by the sheath varies with the medium; sheathed filaments from Knop medium can occupy up to 2½ times the packed-cell volume when transferred to distilled water; unpublished observations.) When the trichome dies, no further sheath material accumulates, and the existing deep layers would be expected slowly to absorb water, expand, become less electron-dense and lose their obvious

striated appearance. This seems to be what happens, because the sheath around old degenerate cells is almost uniformly homogenous and unstriated.

The increase in refractive index of the sheath on mixing cultures with protein solutions at low pH value seems to be in every way comparable with the observations of Tomcsik & Guex-Holzer (1954). These workers demonstrated the precipitation of a variety of proteins on, or within, the capsules of a number of bacteria at pH values near to the isoelectric points of the proteins. The reaction was interpreted as due to the formation of salt-like compounds. Changing the pH resolubilized the protein. In the serological sense these reactions can be interpreted as non-specific capsule-swelling reactions.

Marked sheath production occurred on Knop medium, very little in medium B. The main differences between these media are (*a*) nitrogen source, NO_3^- in Knop, and NH_4^+ in B, and (*b*) there is about three times as much SO_4^{2-} and six times as much PO_4^{3-} in medium B as in Knop medium. What influence the N source may have on capsular (sheath) polysaccharide production cannot be said, but it may be significant that Duguid & Wilkinson (1953) found that deficiency in phosphate or sulphate in the presence of an ample carbon source tended to increase capsule production in *Klebsiella aerogenes*; and with Nostoc FP23, sheath production was much better in the low phosphate medium.

The changes in the sheath next to heterocysts and akinetes are of interest. Both these cells derive from the transformation of single vegetative cells. During the vegetative cell stage incipient heterocysts would be expected to secrete sheath substance, but when they transform to heterocysts extensive changes occur in the cell's anatomy; in particular, the wall or coat becomes greatly thickened, birefringent and accepts Alcian blue (unpublished observations). Metabolic changes probably also occur (Fogg, 1949). Sheath formation ceases, and this would lead to the narrowing and loss of substance frequently seen in sheath adjacent to heterocysts. The function of heterocysts is unknown but in this species, as in many others, filament fragmentation occurs at heterocyst sites and the loss of sheath substance at this point would facilitate separation of the fragments.

Akinetes (spore cells) form by the separation and rounding up of individual vegetative cells. The wall or coat becomes birefringent (unpublished observations), and, as the culture ages, the spores become separated from the parent filament. Subsequently, they germinate, giving rise to new filaments, but, prior to being set free, the sheath substance can be seen to become very loose, frayed and often heavily infested by bacteria. Presumably sheath secretation by incipient akinetes also ceases, and sheath dissolution (aided by bacterial decomposition?) finally releases the spores. These morphological changes undoubtedly reflect switches in cell metabolism accompanying a major cell transformation.

Generally, bacterial capsules are electron-transparent structures, though opaque capsular material has been described in *Mycobacterium tuberculosis* by Knaysi, Hillier & Fabricant (1950). The sheath in Nostoc isolate FP23 is largely electron transparent except in areas where it has probably absorbed relatively little water, i.e. in the striated regions close to the cell wall.

The macro-fibrils noted in a few metal-shadowed filaments have not been identified in sections, but it is emphasized that they were found on only a few filaments, and in these they clearly constituted a small part of the total bulk. Possibly they represent

cellulose fibrils, as have been reported to occur in *Scytonema pseudogyanensi* (Singh, 1954) and *Nostoc* sp. (Frey-Wyssling & Stecher, 1954), but more evidence is needed to confirm this suggestion.

The macro-fibrils show a slightly spiral arrangement with reference to the filament axis, and in some oblique sections the micro-fibril orientation suggests a spiral winding around the trichome. One way such a spiral formation could occur would be for the trichome to move within the sheath. It was noted that trichomes could sometimes be seen to be spirally wound within the sheath (Pl. 1, fig. 2) and probably what has happened in these cases is that the cells have outgrown their sheath and been thrown into a spiral within the sheath. Sections, or metal-shadowed preparations of such a filament, would then show layers of sheath material at an angle to the main filament axis.

The bacteria present in the sections are worth comment. Blue-green algae are notoriously difficult to obtain in axenic cultures. The demonstration of bacteria within the sheath structure points the purely mechanical difficulty of separating the two micro-organisms, but these sections also suggest that there may be some ecological significance in the association. The two organisms are clearly very closely associated and could well exist in nature as a long-lasting partnership.

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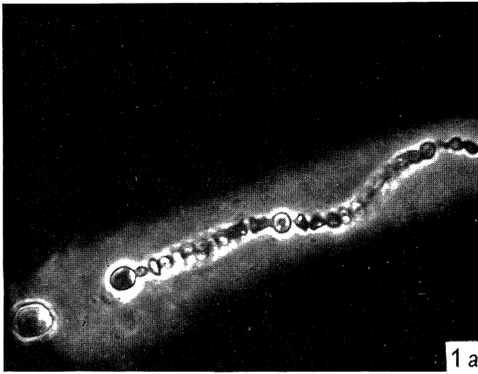

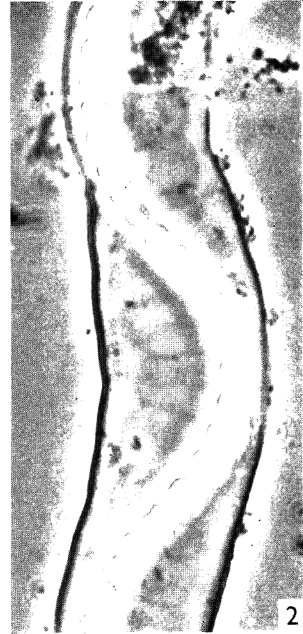

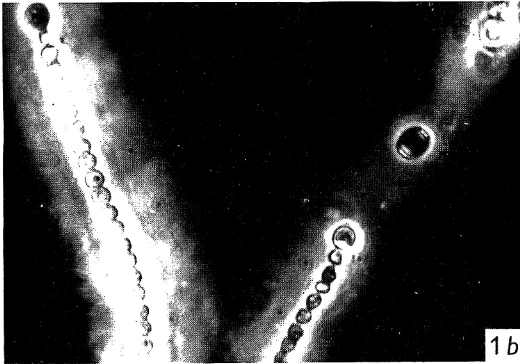

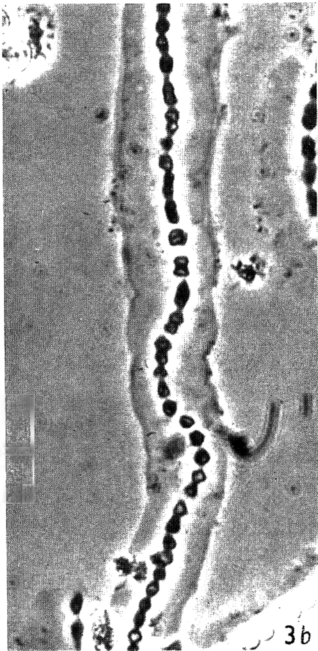

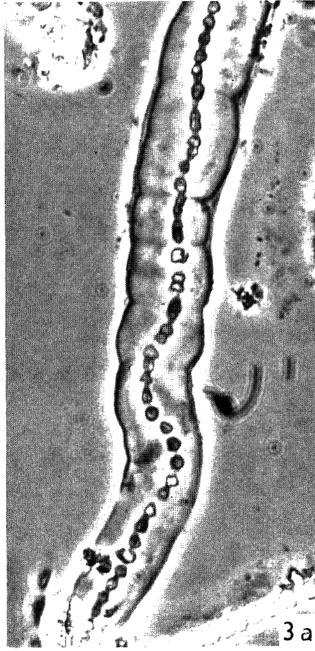

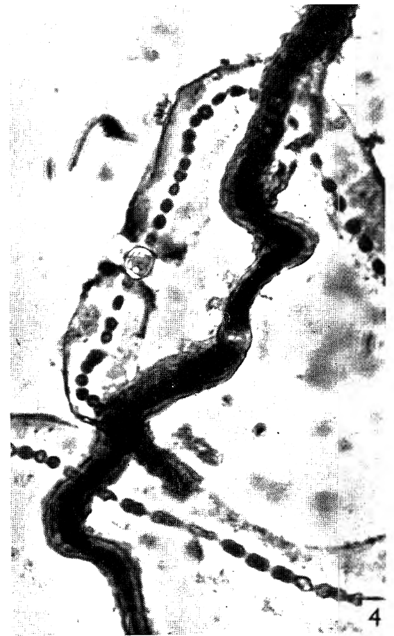

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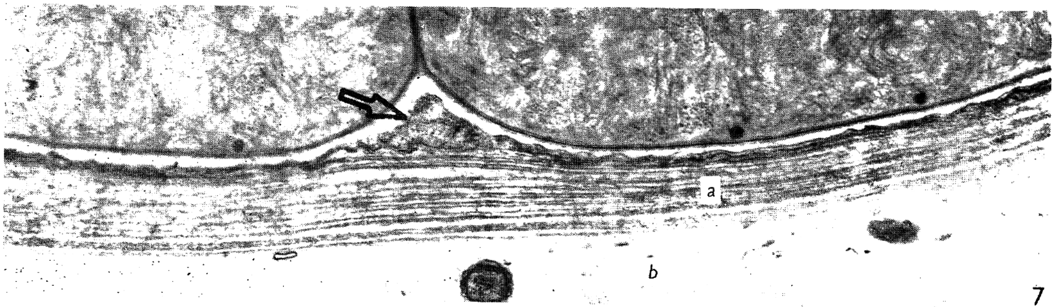

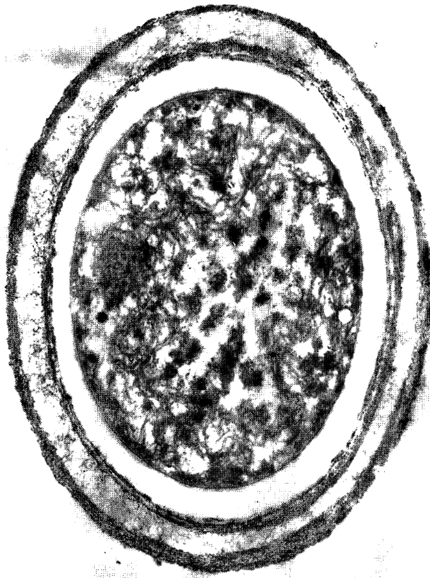

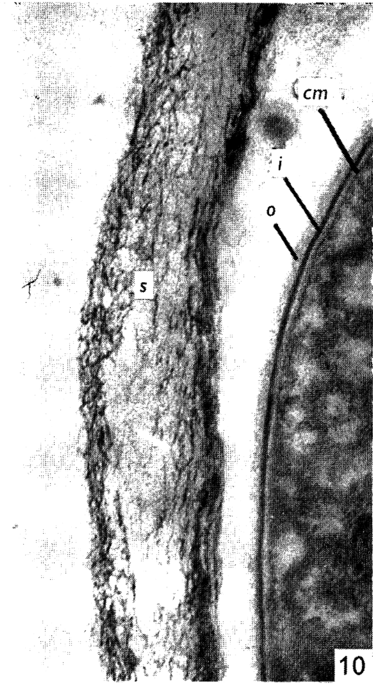

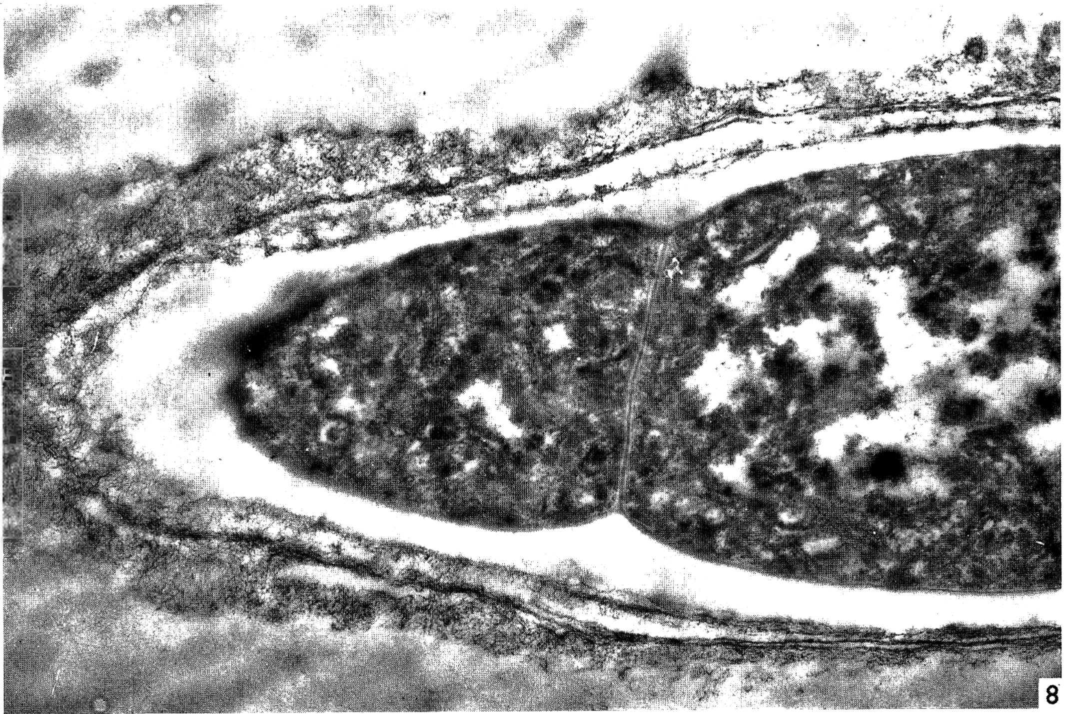

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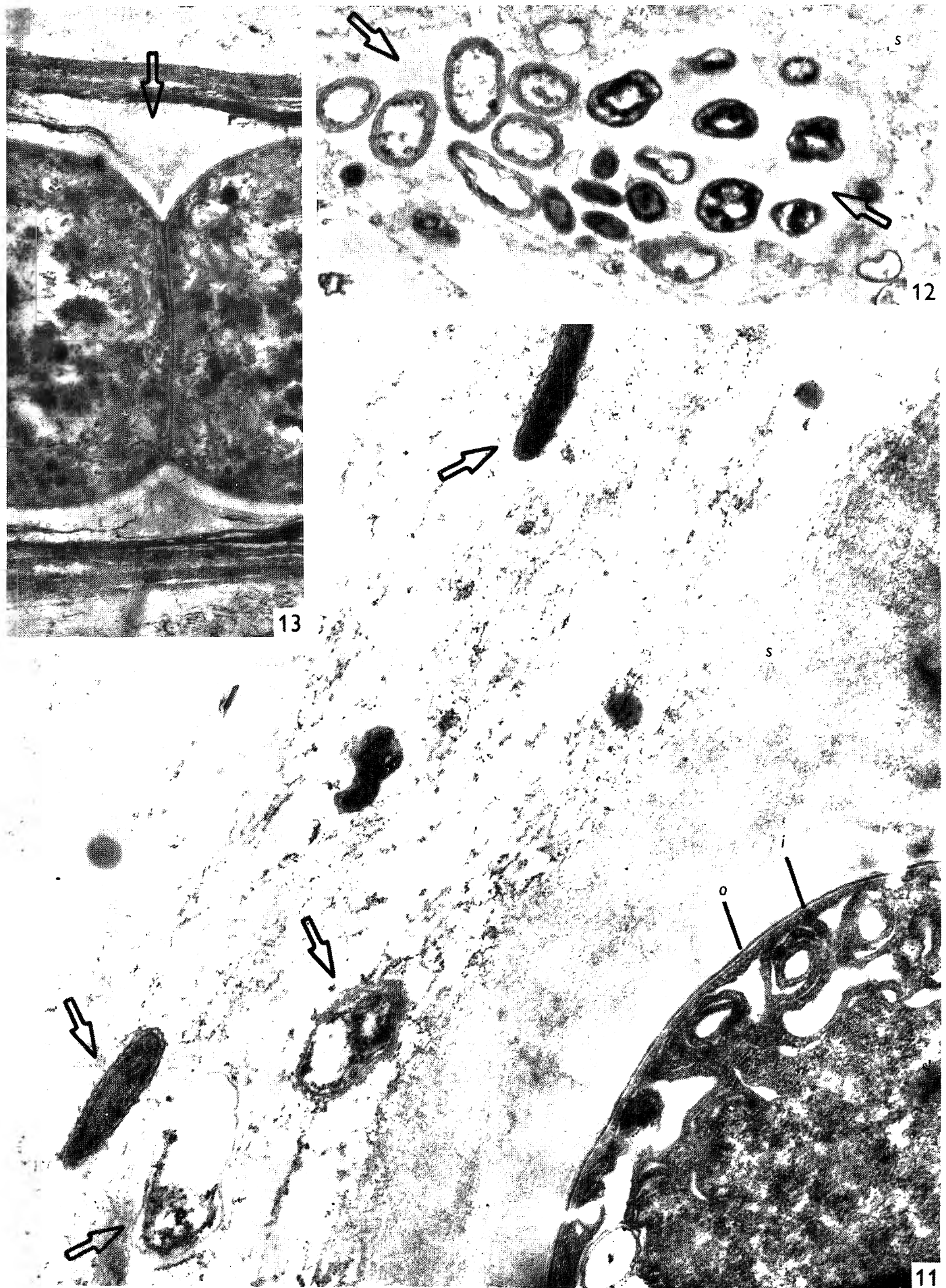

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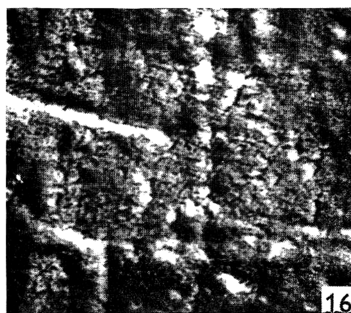

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EXPLANATION OF PLATES

PLATE 1

Fig. 1 to 4 are of unfixed filaments of *Nostoc* sp. isolate FP 23.

Fig. 1 *a*, *b*. Filaments mounted in Indian ink. Note the wide gelatinous fairly sharply delineated sheath around each trichome, which narrows and looses substance next to the heterocysts. Note the clear interval between heterocysts and vegetative cells formed by the degeneration of a number of vegetative cells. × 375.

Fig. 2, 3*a*. Portions of filaments in 0·1 % bovine serum albumin at pH 3·4. Note the intensification of the sheath structure, especially at the edges, and the internal striations. Phase-contrast illumination. Fig. 2 × 560; fig. 3*a* × 400.

Fig. 3*b*. Same field as Fig. 3*a* but the staining with bovine serum albumin has been removed by flushing with buffer at pH 7·2. × 400.

Fig. 4. Filaments mounted in 0·5 % Alcian blue, viewed by phase-contrast illumination. Stain has been taken up by the sheath material only, but neither evenly, nor to the same extent, by all sheaths. × 375.

PLATE 2

Fig. 5 to 16 are of Kellenberger-fixed material cultured in Knop medium except where otherwise noted.

Fig. 5. *Nostoc* sp. Slightly oblique cross-section of a filament. Note compact (*a*) and less compact zones (*b*), showing a fine micro-fibrillar structure. × 26,100.

Fig. 6. Wall structure of *Nostoc* sp. The outer investment is just resolved into a three-layered undulating structure. The inner investment (single dense layer) is visible but the cytoplasmic membrane is not demonstrated. Glutaraldehyde/uranium acetate fixation. × 76,000.

Fig. 7. *Nostoc* sp. Note the compact striations in the sheath close to the cells (*a*) and the much more open network of the micro-fibrils in areas further out (*b*). There is also a much looser, open structure in the groove between separating cells (arrow). A cross-section of a bacterium can be seen embedded in the less compact part of the sheath. × 27,000.

PLATE 3

Fig. 8. *Nostoc* sp. Oblique sections of filament. The more compact layers of the sheath can be seen to consist of fine micro-fibrillar elements. × 23,900.

Fig. 9. *Nostoc* sp. Cross-section of filament, showing micro-fibrillar nature and variations in compactness of the sheath. × 19,500.

Fig. 10. *Nostoc* sp. Cytoplasmic membrane (*cm*); inner investment (*i*) and outer investment (*o*) (poorly visualized with this fixation) of wall; sheath (*s*).

PLATE 4

Fig. 11. *Nostoc* sp. Portion of sheath and cell from an old culture. The sheath striations have completely disappeared, to be replaced by an almost homogenous matrix of sheath substance which became more and more dispersed away from the cell. Bacteria (arrows) are embedded in the outermost regions. Inner investment (*i*), outer investment (*o*), sheath (*s*). × 28,300.

Fig. 12. *Nostoc* sp. Colonies of bacteria embedded in outermost zones of the algal sheaths. Arrows indicate electron-lucent zones of what may be bacterial capsule substance. Algal sheath substance (*s*). × 15,000.

Fig. 13. *Nostoc* sp. The compact layered structure of the sheath gives way to a much looser, open structure in the circumferential grooves or valleys between dividing cells (arrow). × 28,300.

PLATE 5

Fig. 14 and 15. *Nostoc* sp. Portions of trichomes in which the bulk of the sheath substance has dissolved, revealing a loose network of longitudinally orientated macro-fibrils suggestive of cellulose fibrils. Cr shadowed. Fig. 14, × 16,800; fig. 15, × 29,900.

Fig. 16. *Nostoc* sp. Enlargement of arrowed area in fig. 14 showing micro-fibrillar structure of the sheath matrix. Cr shadowed. × 69,000.

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The Electrokinetic Properties of Some Fungal Spores

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SUMMARY

The electrophoretic mobilities of conidia of *Alternaria tenuis*, *Botrytis fabae*, *Penicillium expansum*, *Erysiphe graminis*, *Podosphaera leucotricha* and *Venturia inaequalis*, basidiospores of *Stereum purpureum*, sporangia and encysted zoospores of *Phytophthora infestans* were determined in solution at various pH values. The spores all had characteristic and distinct pH-mobility curves. The zero mobility of *P. infestans* sporangia over the range pH 2 to 11 is consistent with a cellulose surface free from ionizable groups. The mobility of basidiospores of *S. purpureum* depended entirely on the presence of carboxyl groups. Chemical and enzymic treatments showed both amino and carboxyl groups on *A. tenuis* and *B. fabae*; phosphate was present in addition on *P. expansum*. The amino groups of ϵ-lysine, histidine and leucine contributed to the surface charge of *B. fabae*; amino acids and tyrosine were detected on *A. tenuis*. The surface of *P. expansum* was protein-free and the amino groups present were probably derived from a glucosamine or galactosamine polymer. Washed cell walls and intact conidia of *B. fabae* were electrophoretically similar but cell walls of *P. expansum*, unlike normal conidia, were phosphate-free. Mycelial 'protoplasts' of *A. tenuis* and *Neurospora crassa* and conidial 'protoplasts' of *B. fabae* had pH-mobility curves characteristic of a protein surface.

INTRODUCTION

Douglas, Collins & Parkinson (1959) showed that the electrophoretic behaviour of asexual spores from four fungal species indicated marked differences in the chemical nature of the spore surfaces. Hannan (1961) studied the effect of chemical treatments on the mobility of *Aspergillus niger* spores and Somers & Fisher (1967) investigated in detail the elecktrokinetic properties of conidia, protoplasts and cell walls of *Neurospora crassa*. The latter workers found amino, carboxyl and phosphate groups to be present on the spore surface, but phosphate groups were absent from washed spore walls. The cationic surface-active fungicide dodine (*n*-dodecylguanidine acetate) decreased the negative charge on conidia to zero and with increasing concentration gave a positive charge to the spores. The negative charge on cell walls and 'protoplasts' was neutralized at lower fungicide concentrations.

The present work is part of an investigation into the reaction between fungicides and the components of the fungal spore surface. The electrokinetic properties of spores from the following species have been studied to determine the nature of the ionizable surface groups: conidia of *Alternaria tenuis* Nees, *Botrytis fabae* Sardiña, *Penicillium expansum* Link em. Thom, *Erysiphe graminis* DC. ex Mérat, *Podosphaera leucotricha* (Ellis & Everh.) Salm., *Venturia inaequalis* (Cooke) Wint.; basidiospores of *Stereum*

purpureum (Pers. ex Fr.) Fr.; sporangia and encysted zoospores of *Phytophthora infestans* (Mont.) de Bary. The species include such important plant pathogens as the causal organisms of Potato Blight (*P. infestans*), Apple Scab (*V. inaequalis*), Apple Powdery Mildew (*P. leucotricha*), Oat Mildew (*E. graminis*), Silver Leaf of fruit trees (*S. purpureum*) and Chocolate Spot of broad and field beans (*B. fabae*).

METHODS

Fungal material

Conidia from 7-day cultures of *Alternaria tenuis*, *Botrytis fabae* and *Penicillium expansum* were grown and harvested as previously described (Richmond & Somers, 1963). Sporangia of *Phytophthora infestans* were obtained from cultures grown on potato slices (var. King Edward) at 18°. Zoospores were liberated by incubating sporangia in distilled water for 1 hr at 2°. Conidia of the following species were washed from naturally infected material: *Erysiphe graminis* from oat seedlings (var. Black Supreme), *Podosphaera leucotricha* and *Venturia inaequalis* from apple leaves (var. Cox). Basidiospores of *Stereum purpureum* were obtained from fructifications on pear (var. Hendre Huffcap). Protoplasts were prepared from young hyphae of *Alternaria tenuis* and *Neurospora crassa* wild type Em 5297a, and from conidia of *B. fabae* by incubation with *Helix pomatia* digestive-juice extract (Somers & Fisher, 1967). The final protoplast suspension was washed and stabilized in 0·58 M-sucrose maintained at pH 5·6 with sodium acetate buffer (*I*:0·05). Cell walls were obtained by shaking dense spore suspensions at 4° with ballotini (No. 12) in a Mickle disintegrator (Somers & Fisher, 1967). The centrifuged cell walls, which retained the shape of intact conidia, were washed 10 times with 10% (w/v) sucrose, 5 times with 0·9% (w/v) NaCl and 5 times with water following the technique of Dyke (1964).

Electrophoretic measurements

The electrophoretic mobilities of conidia, cell walls and protoplasts were measured by a modification of the technique previously described (Somers & Fisher, 1967). The laterally mounted rectangular cell was enclosed in a water bath maintained at 25·0 ± 0·2°. The water-immersion objective was focused on the stationary layer through a closely fitting rubber sheet. Washed human erythrocytes in 0·067-M phosphate buffer (pH 7·35) were used to calibrate the apparatus (Gittens & James, 1960). The conductivity of the buffered suspensions was measured at 25°, on a Wayne-Kerr B221 bridge. Movement was timed over 180 μ in both directions (current reversal) and each mean mobility was obtained from at least 20 observations: the standard error of the mean was less than 4%. Electrophoretic measurements were made on conidia, cell walls and protoplasts (1 million/ml.) which had been washed twice with the appropriate buffer before suspension in HCl + NaCl or barbiturate + acetate buffer (*I*:0·05) of the required pH (Gittens & James, 1960). Protoplasts were measured in buffer containing 0·58 M-sucrose.

Treatments to modify surface groups

Alkaline phosphatase: (EC 3.1.3.1). Washed conidia and cell walls were suspended in barbiturate buffer (pH 7·9, *I*:0·02) containing 5 μg. alkaline phosphatase/ml. for 1 hr at 37° (Hill, James & Maxted, 1963).

1-Fluoro-2,4-dinitrobenzene (*FDNB*). Conidia and cell walls were washed 3 times in

phosphate buffer (pH 7·0, *I*:0·05) then suspended in an 0·1 % (v/v) ethanolic FDNB solution containing 0·9% (w/v) $NaHCO_3$ for 5 hr (Gittens & James, 1963). The sediment was washed 5 times with ethanol before washing with the final buffer solution.

p-Toluenesulphonyl chloride (PTSC). Washed conidia in barbiturate buffer (pH 7·0, *I*:0·05) were shaken with 50 mg. PTSC for 24 hr at room temperature (Gittens & James, 1963).

Diazomethane (DAM). Washed conidia and cell walls were methylated as previously described (Somers & Fisher, 1967). Conidia were washed once with phosphate buffer (pH 7·0) and twice with HCl (0·05 M) before methylation.

Identification of N-terminal amino acid groups

Conidia after treatment with FDNB were hydrolysed in a sealed tube at 105° for 16 hr in the presence of 5·7 N-HCl. After extraction (Biserte, Holleman, Holleman-Dehove & Sautière, 1960) the DNP-amino acids were separated by thin layer chromatography (Pataki, 1967).

Lipid determination

Total lipid was determined by extraction of dried cell walls (*in vacuo* over P_2O_5) with hot chloroform + methanol (2 + 1, v/v) followed by hot ether.

RESULTS

Electrophoretic properties of fungal conidia. Sporangia of *Phytophthora infestans* had zero mobility over the whole pH range (Fig. 1), indicating the absence of surface ionogenic groups. Zoospores of *P. infestans* (Fig. 1) and basidiospores of *Stereum purpureum* (Fig. 2) showed no positive mobility at low pH, suggesting a preponderance of acidic surface groups. The pH-mobility curve of *S. purpureum* is characteristic of a simple carboxyl surface (Hill *et al.* 1963). Cells treated with alkaline phosphatase had a similar mobility to untreated spores, confirming the absence of phosphate groups. Treatment with DAM (Fig. 2) decreased the mobility to zero between pH 2 and 5, suggesting that only carboxyl groups contribute to the surface charge. Above pH 7, however, the mobility did not return to its original value after hydrolysis of the methyl esters. DAM treatment may block other surface groups. Suspension in 0·05 M-HCl followed by ether + ethanol and ethanol as required for the chemical treatments did not cause irreversible changes to any of the spore surfaces. After resuspension in pH 7 buffer mobilities were the same as normal control spores.

The pH/mobility curves of *Podosphaera leucotricha*, *Erysiphe graminis*, *Venturia inaequalis*, *Alternaria tenuis* and *Botrytis fabae* (Fig. 2 to 5) are characteristic of mixed aminocarboxyl surfaces (James & List, 1966).

Treatment of *Alternaria tenuis* and *Botrytis fabae* (Fig. 4 and 5) with FDNB decreased the positive mobility at low pH and removed the inflexion at pH 10·0, confirming the presence of amino groups on the untreated surface. Hydrolysis of FDNB-treated *B. fabae* conidia followed by chromatography revealed spots corresponding to the DNP derivatives of ε-lysine, histidine, leucine and an unidentified compound; *A. tenuis* conidia revealed, in addition, a spot corresponding to DNP-tyrosine. The unidentified compound in *A. tenuis* and *B. fabae* had a similar R_F value

to the DNP derivatives of D-glucosamine and D-galactosamine, and might have been derived from a glucosamine or galactosamine polymer in the cell wall.

Cell walls of *Botrytis fabae* gave a pH/mobility curve of the same general shape and with the same isopotential point (3·8) as intact conidia. Conidia of *Alternaria tenuis*, unlike the other spores examined, are multicellular; this made it difficult to prepare clean cell walls which still retained the shape of intact conidia. Methylation of *A. tenuis* conidia (Fig. 4) and *B. fabae* walls (Fig. 5) with DAM decreased the negative mobility by removing the charge on the carboxyl groups; positive mobilities below

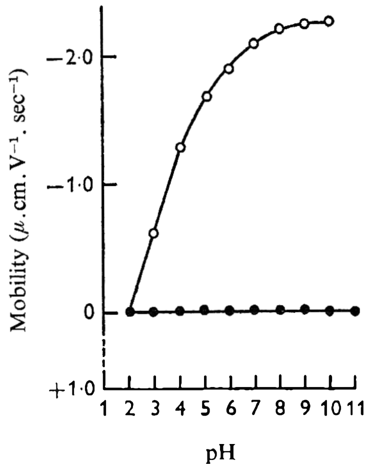

Fig. 1

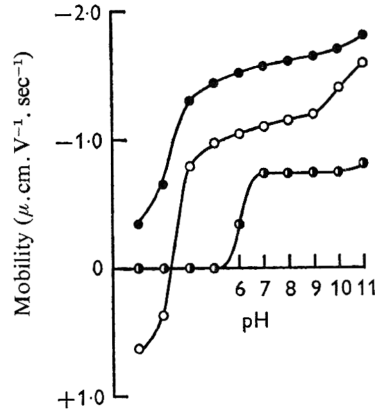

Fig. 2

Fig. 1. pH/mobility curves of *Phytophthora infestans* sporangia, ●—●; encysted zoospores, ○—○.

Fig. 2. pH/mobility curves of conidia of *Venturia inaequalis*, ○—○; basidiospores of *Stereum purpureum*, ●—●; and DAM-treated basidiospores of *S. purpureum*, ◑—◑.

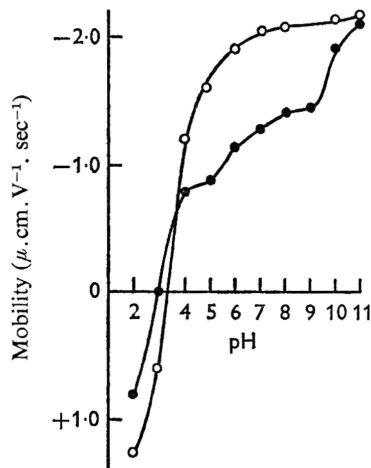

Fig. 3. pH/mobility curves of conidia of *Podosphaera leucotricha*, ●—●; and *Erysiphe graminis*, ○—○.

6·0 are due to the remaining amino groups. The decrease in positive mobility of DAM-treated cells below pH 4 may be due to some interaction with amino groups. Mobilities were unaffected by treatment with alkaline phosphatase. Only small increases in the mobility of conidia or cell walls occurred in the presence of sodium dodecyl sulphate (SDS), showing the absence of appreciable amounts of surface lipid (Table 1). The

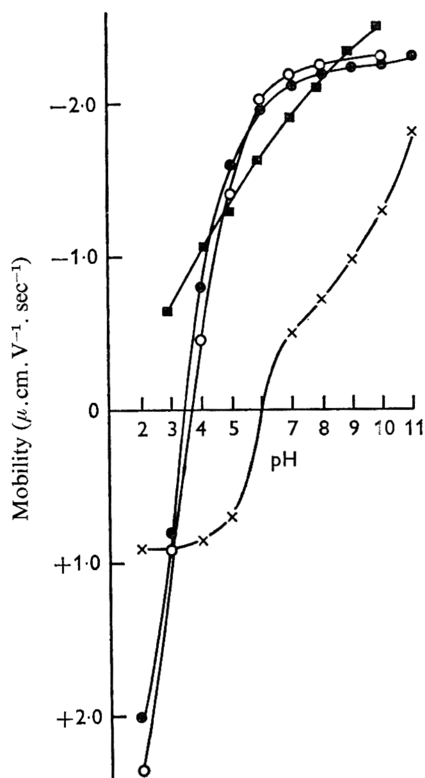

Fig. 4

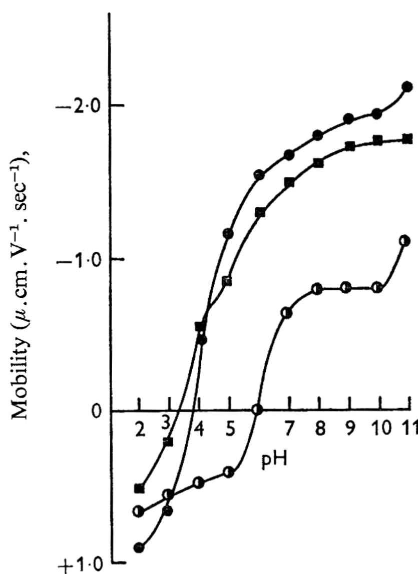

Fig. 5

Fig. 4. pH/mobility curves of conidia of *Alternaria tenuis*. Untreated, ●—●; phosphatase-treated, ○—○; FDNB-treated, ■—■; DAM-treated, ×—×.

Fig. 5. pH/mobility curves of *Botrytis fabae*. Untreated conidia, ●—●; FDNB-treated conidia, ■—■; DAM-treated cell walls, ◐—◐.

Table 1. *Effect of sodium dodecyl sulphate (SDS) on the electrophoretic mobility of conidia and washed cell walls*

Suspension medium: phosphate buffer solution (pH 7·0, *I*: 0·01)

Material	SDS concentration (M) 0	10^{-6}	10^{-5}	10^{-4}
	Electrophoretic mobility μ. cm. V^{-1}. sec^{-1}			
A. tenuis (conidia)	−2·62	−3·02	−3·04	−3·26
B. fabae (walls)	−1·41	−1·45	−1·49	−1·53
P. expansum (conidia)	−1·54	−1·80	−1·76	−1·76
P. expansum (walls)	−1·52	−1·42	−1·57	−1·56

total lipid content of cell walls on a dry weight basis was 4·6 % for *A. tenuis* and 6·6 % for *B. fabae*. These figures are much lower than the value of 14 % reported for *Neurospora crassa* (Somers & Fisher, 1967).

The pH/mobility curve for *Penicillium expansum* (Fig. 6) with an isopotential point at pH 2·0 suggests the presence of highly acidic phosphate groups; treatment with alkaline phosphatase (Fig. 6) revealed an underlying amino-carboxyl surface with an

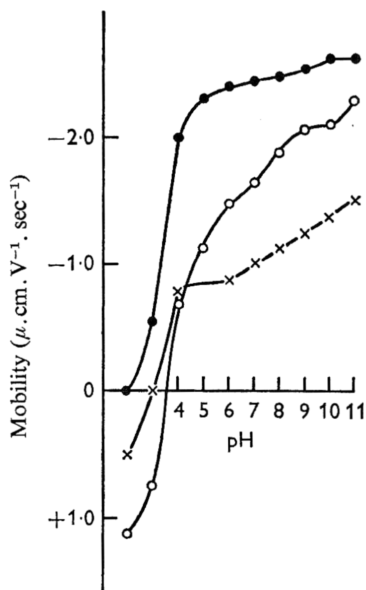

Fig. 6

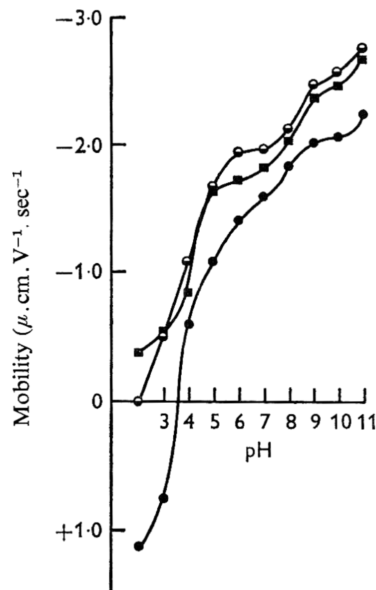

Fig. 7

Fig. 6. pH/mobility curves of conidia of *Penicillium expansum*. Untreated, ●—●; phosphatase-treated, ○—○; DAM-treated, ×—×.

Fig. 7. pH/mobility curves of washed conidia of *Pencillium expansum*, Untreated, ●—●; FDNB-treated, ■—■; PTSC-treated, ⊖—⊖.

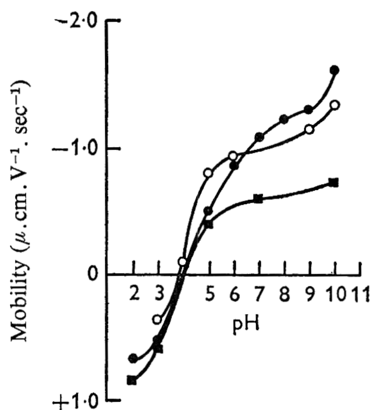

Fig. 8. pH/mobility curves of fungal protoplasts. Hyphal protoplasts of *Neurospora crassa*, ●—●; hyphal protoplasts of *Alternaria tenuis*, ■—■; conidial protoplasts of *Botrytis fabae*, ○—○.

isopotential point of 3·5 similar to untreated *Alternaria tenuis* and *Botrytis fabae* (Fig. 4 and 5). Additional evidence for the presence of phosphate groups on intact *P. expansum* conidia was provided by the decrease in mobility at pH 7 in the presence of 0·01 M-Ca^{2+} from $-2{\cdot}40$ to $-1{\cdot}60$ μ.cm.V^{-1}. sec^{-1}. The washing technique of Dyke (1964), when applied to intact conidia of *P. expansum* (Fig. 7), was as effective as alkaline phosphatase in removing phosphate groups, but extraction with chloroform + methanol (2 + 1, v/v) had no effect on the isopotential point, showing that the phosphate was not present as phospholipid. Indeed, the total lipid content of cell walls was only 1·1 % on a dry weight basis, and mobility measurements in the presence of SDS (Table 1) showed that none of this was present on the surface of either intact conidia or washed cell walls.

Conidia of *Penicillium expansum* treated with DAM (Fig. 6) showed decreased negative mobility due to removal of the charge on the carboxyl groups; after hydrolysis of the methyl ester the mobility was still lower than that of untreated conidia, suggesting that DAM treatment had some blocking action on the amino groups. Interpretation of the pH/mobility curve obtained from FDNB-treated conidia was complicated by the simultaneous removal of some of the labile phosphate component. However, when conidia, washed free of phosphate, were treated with FDNB (Fig. 7) the negative mobility was increased, confirming the presence of amino groups. The inflexion at pH 7 to 8 was not due to secondary amino groups, as it was still present when washed conidia were treated with PTSC (Fig. 7), a reagent which reacts with both primary and secondary amines (Gittens & James, 1963).

Hydrolysis of FDNB-treated conidia followed by chromatography revealed one spot with the same R_F value as the unidentified compound found in *Alternaria tenuis* and *Botrytis fabae*. No spots corresponding to DNP-amino acids were produced, indicating a probable absence of surface protein.

Electrophoretic properties of protoplasts. The pH/mobility curves of protoplasts stabilized in 0·58 M-sucrose from mycelium of *Neurospora crassa* and *Alternaria tenuis* and from conidia of *Botrytis fabae* (Fig. 8) have isopotential points (3·9 to 4·0) typical of a protein surface. (Protoplasts from *B. fabae* represent only a part of the cell contents, being liberated from the germ tube of germinating conidia by constriction of portions of the protoplasm.)

DISCUSSION

All the fungal spores examined have characteristic and distinct electrophoretic behaviours. The sigmoid pH-mobility curves given by protoplasts of *Alternaria tenuis*, *Botrytis fabae* and *Neurospora crassa*, and by conidia of *A. tenuis*, *B. fabae*, *Erysiphe graminis* and *Podosphaera leucotricha* are typical of amino-carboxyl surfaces. Conidia of *Penicillium expansum*, *Stereum purpureum* and encysted zoospores of *Phytophthora infestans* show no positive mobility at low pH, indicating a preponderance of acidic surface groups. Sporangia of *P. infestans* are unusual in having zero mobility over the whole pH range and must be completely lacking in surface ionizable groups. Clean *B. fabae* cell walls are electrophoretically similar to intact conidia but *P. expansum* walls are quite distinct from *P. expansum* conidia: the washing technique removed phosphate groups from *P. expansum* conidia to reveal an underlying amino + carboxyl surface. Chemical treatment of *A. tenuis* and *B. fabae* has shown phosphate and lipid

to be absent from the surface and confirmed the presence of amino and carboxyl groups.

The amino groups on the spore surfaces are probably a part of the protein components of the cell wall (Aronson, 1965; Manocha & Colvin, 1967), although aminopolysaccharides (Harold, 1962) may also be present. Carboxyl groups may derive from proteins, polysaccharides (Applegarth, 1967) or uronic acids (Mitchell & Scurfield, 1967). Somers & Fisher (1967) found ϵ-lysine and histidine on the surface of *Neurospora crassa* conidia and we have found, in addition, leucine on *Botrytis fabae*, and leucine and tyrosine on *Alternaria tenuis*. The high positive mobility of *A. tenuis* at low pH together with the negative mobility after treatment with FDNB suggests that *A. tenuis* has a higher proportion of surface amino groups than *B. fabae*.

The surface of *Penicillium expansum* resembles that of *Neurospora crassa* (Somers & Fisher, 1967) in having easily removable phosphate groups but differs in having no surface lipid. This is reflected in the much lower total lipid content of cell walls of *P. expansum* (1·1 %) compared with 14 % reported for *N. crassa* by Somers & Fisher (1967). Although no DNP-amino acids were detected in acid hydrolysates from FDNB-treated conidia an unidentified compound was found which could have been the DNP-derivative of glucosamine or galactosamine. This suggests that the surface is protein free but may contain polyglucosamines or polygalactosamines. Harold (1962) has shown that inorganic polyphosphate can bind to protein and polygalactosamine receptor sites on the outer surface of *N. crassa* hyphae: the phosphate groups on *P. expansum* conidia may be bound in a similar way. The observation of Hess, Sassen & Remsen (1968) that a very thin layer is present on the outside of conidia of some Penicillium species when examined by carbon replica techniques may also be relevant here.

As the only ionized groups on *Stereum purpureum* are carboxyl the surface must be protein-free and probably consists entirely of carbohydrate. The absence of ionogenic groups from sporangia of *Phytophthora infestans* is consistent with the work of Chapman & Vujičić (1965), who showed that young sporangia of *Phytophthora erythroseptica* have a structureless, electron-transparent outer layer consisting probably of cellulose.

The protein-like pH/mobility curves of the fungal protoplasts are in agreement with the lipoprotein constitution of fungal cytoplasmic membranes (Villanueva, 1966). The curves are similar to those reported for rat liver nuclei by Vassar, Seaman, Dunn & Kanke (1967) and bacterial protoplasts by James, Hill & Maxted (1965) in conformity with the unit membrane theory (Robertson, 1959).

The water-repellent properties of Penicillium conidia have been attributed to the presence of ether-soluble cyclic peptides on the spore surface (Bertaud, Morice, Russell & Taylor, 1963) or to the characteristic 'rodlets' which may consist of cutin or sporopollenin detected on the surface of freeze-etched conidia by Hess *et al.* (1968). The small amount of material soluble in chloroform + methanol in cell walls and the absence of surface lipid suggest that the water-repellent properties are not due to lipids or cyclic peptides. Further studies in conjunction with chemical treatments are required to elucidate the problem. Powdery mildew conidia are also water-repellent and differ from spores of other fungi in their resistance to desiccation (Yarwood, 1936) furthermore spore germination is usually impaired by wetting (Zaracovitis, 1964). McKeen, Mitchell & Smith (1967), in a study of the *Erysiphe cichoracearum*

conidium, considered that the impervious outer layer of the spore played an important role in the action of water and fungicides on mildew conidia. The pH-mobility curves of *Podosphaera leucotricha* and *Erysiphe graminis* do not account for the characteristic physical properties of powdery mildew conidia: unionized compounds may be responsible for the non-wettable nature of these spores.

Fungal spores frequently have a specialized and complex morphology which differs considerably from the vegetative cells. Electron microscopic studies of germinating spores have shown that the germ tube wall may be continuous with either the inner (Remsen, Hess & Sassen, 1967), or the outer (Manocha & Shaw, 1967) layer of the spore wall, or an entirely new wall may be formed (Hawker, 1966). Several detailed studies have been made of the chemical composition of hyphal cell walls (Aronson, 1965; Rogers & Perkins, 1968), but less attention has been paid to the spore wall, although Horikoshi & Iida (1964) have compared the composition of hyphal and conidial walls of *Aspergillus oryzae* and detailed studies have been made of the chemical structure of the cell wall of *Mucor rouxii* at different stages of development (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia & Reyes, 1964; Bartnicki-Garcia & Reyes, 1968). Spore walls and hyphal walls of *A. oryzae* were qualitatively identical but the composition of spore walls of *M. rouxii* was markedly different from vegetative cell walls. Differences in cell wall composition may explain why fungal spores differ in their susceptibility to fungicides and why some fungicides are more effective against spores than mycelium, while, with others, the reverse is true (Kreutzer, 1963). The physical properties of the spore surface will influence dispersion by air, water or animals (Gregory, 1966); the chemical composition may modify toxicant action. Studies of the fungal spore surface may make some contribution to an understanding both of fungal ecology and of the mechanisms of the selective toxicity of compounds to spores of different fungal species as well as to spores and mycelium of the same species.

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The Biochemical Status of μ Particles in *Paramecium aurelia*

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SUMMARY

μ particles of stock 540 (syngen 1) of *Paramecium aurelia* were isolated and studied. Electron microscopy showed that isolated *μ* particles were very similar to bacteria in fine structure, the general appearance being similar to that found by earlier workers. Contamination of the preparations with other cell particles and with the paramecium food bacteria was low. The isolated *μ* particles contained DNA, RNA, protein and probably carbohydrate and lipid. The DNA of the particles was characterized by density-gradient centrifugation in CsCl and by determination of Tm. The density of the DNA was estimated to be 1·694 g./c.c., a value very similar to that for the DNA of *Paramecium aurelia* 540 macronuclei, which was estimated to be 1·693 g./c.c. The Tm was likewise similar, being 82·0°, against 81·8° for Paramecium macronuclear DNA. However, this DNA was associated with the *μ* particle, as no such DNA could be prepared from genotypically identical *P. aurelia* which did not possess *μ* particles, and the DNA associated with the *μ* particles was not susceptible to DNase action on treatment of isolated *μ* particles with the enzyme before DNA extraction. Hence it seems that the *μ* particle has a membrane-protected DNA of a similar buoyant density to the *P. aurelia* DNA. Isolated *μ* particles possessed an active DNA-dependent system capable of the incorporation of ribonucleoside triphosphates into an acid-insoluble product. The reaction needed the presence of ATP, GTP, and UTP for optimal activity, and had an absolute dependence on Mg^{2+}. The reaction was inhibited by actinomycin D, and a ribonuclease-sensitive product was synthesized.

INTRODUCTION

Many stocks of the ciliate *Paramecium aurelia* have been observed to possess cytoplasmic particles. The possession of these particles may be correlated with a killing or mate-killing ability of the bearer animal upon stocks of paramecia that do not bear particles. These particles have been the subject of much experiment and speculation, and a general conclusion has been reached that they are bacterium-like endosymbiotes (Sonneborn, 1959; Beale & Jurand, 1960, 1966; van Wagtendonk, Clark & Godoy, 1963).

The *μ* particle of stock 540 (syngen 1) of *Paramecium aurelia* was studied by Beale & Jurand (1960) by electron microscope and cytochemical techniques; their results clearly indicate that this particle may be an endosymbiotic bacterium. Van Wagtendonk, Clark & Godoy (1963) succeeded in culturing the lambda particle of stock 299 (syngen 4) in a complex medium outside the host cell, while van Wagtendonk & Tanguay (1963) showed that DNA, RNA, protein, carbohydrate and lipid were present in isolated lambda particles.

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The most extensively studied particle, the kappa particle of stock 51 (syngen 4), resembles a bacterium in many respects, but shows some unusual features; it has two forms, bright (B) particles containing a refractile or R body, and non-bright or N particles. Kappa of stock 7 (syngen 2) also seems like this. B particles are responsible for the killing effect, while N particles are the reproductive body (Smith, 1961; Mueller, 1962). In the electron microscope, the R body has the appearance of a tightly coiled roll which can suddenly and quickly unroll to form a twisted ribbon about 15 μ long (Anderson, Preer, Preer & Bray, 1964; Preer, Hufnagel & Preer, 1966). Possession of this singular body would seem to distinguish the kappa particles of stocks 51 and 7 from the other particles, in which no such structures have been observed.

In the present paper, results of some studies on isolated μ particles of stock 540 are presented which provide further evidence for the bacterial nature of this particle. A partial account of this work has previously been published (Stevenson, 1967*a*), and an abstract presented (Stevenson, 1967*b*).

METHODS

Stocks of Paramecium used and culture medium

The following stocks of *Paramecium aurelia* were used: stock 540, bearing μ particles (mate-killer); stock 540, not bearing μ particles (sensitive), derived from stock 540 bearing μ particles by prolonged culture at 31°. The presence or absence of μ particles is the sole difference between these two stocks. Genetically they are identical.

Paramecia were maintained at 18° in 1 l. batches of lettuce extract medium (pH 6·8) in 3-l. flat culture flasks. The lettuce extract was prepared as described by Sonneborn (1950). Paramecia were subcultured every 3 weeks. This method was found to be more reliable for prolonged particle maintenance than slide-cultures, and decreased the labour involved in setting up large cultures. Autogamy was induced at 3-month intervals, and fresh maintenance cultures set up from ex-autogamous clones.

Mass cultures were grown and harvested as described by Jones (1965), except that growth was at 25° for 5 days; 50 l. of culture were normally grown. After harvesting, the paramecia were washed twice with the solution described by Dryl (1959).

Paramecia were tested for particles at all stages of growth, and at subculture and autogamy, by the method described by Beale & Jurand (1966).

Isolation of μ particles

Washed paramecia were suspended in 4 volumes of ice-cold Dryl solution and homogenized by using a Tri-R stirring motor (Tri-R Instruments, Jamaica, New York, U.S.A.), for 20 to 30 strokes of a teflon pestle. Homogenization was examined under a binocular microscope; it was considered complete when fewer than 1 % whole paramecia were left. The homogenate was centrifuged at 35,000 *g* for 7 min. (MSE HS 18 centrifuge) and the pellet was suspended in 40 ml. of 0·01 M-Na phosphate (pH 6·8). The supernatant fluid was discarded.

Body and cell-wall fragments, trichocysts and many cilia were removed by passing the suspension of pellet material through a column of cellulose (Preer *et al.* 1966). During the preparation and centrifugation of the homogenate, 15 to 20 g. of cellulose (Whatman cellulose powder CF 11) were stirred into 200 ml. of the phosphate buffer

and then poured into a 40 cm. × 2·5 cm. chromatography column. The column of cellulose was washed with 200 ml. of buffer. After draining to bed level, the re-suspended 35,000 *g* pellet was rapidly applied by using air pressure, then fresh buffer was added above the cellulose, and the column was washed with further buffer, using a peristaltic pump to speed the flow. The eluate was collected till it was no longer visibly turbid. On examination in the phase-contrast microscope it was seen to contain mitochondria, a small number of cilia, a few *Aerobacter aerogenes* and the μ particles. Usually, about 5×10^8 μ particles/ml. were present, with a similar or rather larger number of mitochondria.

The eluate was centrifuged at 22,000 *g* for 15 min. and the pellet suspended in 9 ml. of the phosphate buffer; 3 ml. of this suspension were layered over a 10 to 40 % (w/v) gradient of Ficoll (Pharmacia, Uppsala, Sweden) in a thin-walled polypropylene centrifuge tube. Gradients were 32 ml. and were prepared by layering 10, 20, 30 and 40 % Ficoll in the tubes 18 to 24 hr before use, then keeping at 4°. Three gradients were normally run.

Gradients were centrifuged at 10,000 rev./min. (10,500 *g* av.) in the 3 × 40 ml. swing-out rotor of the MSE 'Superspeed 50' ultracentrifuge, for 45 min. After centrifugation the particles could be seen as a distinct band in the gradients. This band was only present in preparations from stock 540 mate-killers; preparations from stock 540 sensitive did not show it.

Gradients were fractionated with a 5-ml. syringe and blunt-tipped 18 G. needle (5 cm.). Six fractions of 3 ml. each were removed. The fractions were examined at × 800 under phase-contrast; μ particles, when present, were in fraction 4 from the top. The fractions bearing μ particles were pooled and centrifuged at 38,000 *g* for 10 min. to pellet the μ particles. The pellet was washed once with phosphate buffer and recentrifuged. Finally the pellet was washed with distilled water or an appropriate buffer.

With the exception of the cellulose column step, which was done at about 20°, all preparative steps were at 0 to 4°.

Techniques for electron microscopy

μ particles were prepared for electron microscopy by a method very similar to that of Kellenberger, Ryter & Séchaud (1958). μ particles were fixed in veronal + acetate buffered $OsO_4 + CaCl_2$ to 0·001 M, the fixative given by these authors. The particles were embedded in 2 % (w/v) agar, washed in the veronal + acetate buffer + uranyl acetate, dehydrated through ethanol and embedded in Araldite. Sections were cut at 500 to 800 Å with a Porter–Blum Servall microtome, the sections were stained with lead citrate (Reynolds, 1963) + 2 % (w/v) uranyl acetate and then examined with either a Philips EM 75 or AEI EM 6 electron microscope.

Chemical methods

DNA was estimated by the diphenylamine reaction as modified by Burton (1956). RNA was estimated by the orcinol reaction (Ogur & Rosen, 1950) and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Carbohydrate was estimated by the anthrone reaction and lipid by the silver dichromate reaction (Bloor, 1947). Lyophilized material was used as starting material for these assays.

Preparation of DNA

DNA for use as standards was prepared from spray-dried *Micrococcus lysodeikticus* (Cambrian Chemical Co., London, S.E. 16), from *Aerobacter aerogenes* strain A 3(o) (a rough strain used as food organism for the Paramecium in all these experiments) and from isolated macronuclei of *Paramecium aurelia*. The *A. aerogenes* was obtained from Dr J. F. Wilkinson, Dept. of Bacteriology, University of Edinburgh. The macronuclei were isolated as described by Stevenson (1967*c*). DNA was also prepared from isolated μ particles.

To prepare DNA from *Micrococcus lysodeikticus*, 10 g. dried bacteria were suspended in 45 ml. of 0·15 M-NaCl+0·15 M-sodium citrate (SSC) by stirring for 4 hr at 4°. The suspended bacteria were treated with lysozyme (EC 3.2.1.17; 0·1 mg./ml. Sigma) at 37° until lysis occurred (a great increase in viscosity), then sodium dodecyl sulphate (SDS) was added to a final concentration of 0·3 %, and this mixture was kept at 60° for 10 min. After cooling, Pronase (Calbiochem) was added to 0·2 mg./ml., and the mixture incubated at 37° overnight. The Pronase was self-digested for 2 hr before use. The digest was deproteinized with 9+1, v/v, chloroform+octanol mixture and the procedure of Marmur (1961) followed. The final preparation was stored in the refrigerator over chloroform.

DNA was prepared from exponential phase *Aerobacter aerogenes* A 3(o). Bacteria were grown in a glucose+salts medium to an extinction at 650 mμ of 1·0, harvested by centrifugation at 7,000 *g* for 10 min. at 3°, washed once with 0·15 M-NaCl+0·01 M-EDTA (pH 8·0) and centrifuged down. DNA was prepared by the method of Marmur (1961) and stored as before.

Isolated macronuclei of *Paramecium aurelia* were resuspended in SSC to a volume equal to that of the original packed paramecia from which they were prepared, and SDS was added to 0·1 %. The lysate was kept at 65° for 20 min., allowed to cool and then Pronase was added to 0·1 mg./ml. After overnight digestion at 37° the extract was deproteinized three times with 9+1 (v/v) chloroform+octanol mixture. This final extract was then subjected to a CsCl gradient centrifugation.

Pronase digestion followed by deproteinization was unsatisfactory for the preparation of DNA from μ particles. It was found better to suspend the isolated particles in 1·5 ml. of 4×SSC and freeze at −20°. After thawing, 0·5 ml. of 10 % SDS was added, followed by treatment at 65° for 20 min. After cooling, the volume was made to 3 ml. with 4×SSC, and a CsCl gradient was carried out. CsCl for use in gradients was purified by precipitation with ethanol and washing with ether. Background absorption at 260 mμ was 0·02 or less at the concentration used.

To run gradients, 3·40 g. of CsCl were dissolved in 2·5 ml. of the DNA preparation and this solution poured into a 5-ml. thin-walled polypropylene tube, and 1 ml. of liquid paraffin was overlaid. The 3×5 ml. swing-out rotor of the MSE 'Superspeed 50' ultracentrifuge was used, centrifugation being for 65 to 75 hr at 32,000 rev./min. at 18 to 20°. The rotor was allowed to coast to a stop, and gradients were fractionated by collecting drops. To each fraction was added 0·8 or 1·0 ml. of SSC, and the extinction at 260 mμ was read in a Beckman DB spectrophotometer, with 0·5 cm. light-path optical cells. Sometimes gradient fractions were assayed for DNA content by the diphenylamine method (Burton, 1956). Material to be run in a gradient was always assayed by this method to determine the amount of DNA present. Tm was determined as described by Marmur & Doty (1959) but a Beckman DB spectrophotometer was used

Assay for ribonucleoside triphosphate incorporation

The assay mixture was similar to that of Weiss (1960) except that either (8-^{14}C) ATP or (4-^{14}C) UTP (from the Radiochemical Centre, Amersham, Bucks., England) or GTP-^{3}H (obtained from Schwarz Bioresearch Inc., Orangeburg, New York, U.S.A.) were the radioactive triphosphates used in the assay. Incubation, precipitation, sample preparation and counting procedures followed those described earlier (Stevenson, 1967*c*).

RESULTS

General observations on isolated μ *particles*

The preparations obtained from *Paramecium aurelia* stock 540 bearing μ particles consisted of at least 90% of rod-shaped particles 2 to 5 μ long × 0·5 μ wide. Such particles were not obtained in preparations from paramecia not bearing μ particles. Phase-contrast microscopy showed the presence of contaminating cell debris in μ particle preparations; but this debris was the only material obtained in preparations from the paramecia not bearing μ particles. Electron microscopy of the μ particle preparations showed that the principal contaminants were cilia and fragments of cilia, some mitochondria and other small debris (Pl. 1, fig. 1). The appearance of the isolated μ particle compares well with the appearance found by Beale & Jurand (1960, 1966), but the capsule reported by these authors was not observed. The presence of this component appeared to be variable. Other electron micrographs showed μ particles which had a median constriction; these may have been in the course of division.

Table 1. *Contamination of* μ *particle and control preparations with viable Aerobacter aerogenes A3(o)*

The left-hand column is a total count of particles of all kinds in a preparation, i.e. μ particles, mitochondria, *Aerobacter aerogenes* and others. Each preparation was made to 10 ml. before the haemocytometer count, and all preparations were made from paramecia which had been grown in 50 l. culture medium. The three upper lines refer to experiments with paramecia of *Paramecium aurelia* stock 540 bearing μ particles; the fourth line is a control experiment made with *P. aurelia* stock 513 (sensitive paramecia). Suspensions were serially diluted to 10^{-4}, and 0·1 ml. was plated in triplicate on to nutrient agar plates, and the mean number of bacterial colonies after 60 hr of incubation at 35° taken. Shorter incubation periods did not show the true extent of the bacterial contamination.

Haemocytometer count no. of particles/ml. suspension	Dilution for plating	No. bacterial colonies/ml. after 60 hr at 35° (mean)	Viable *A. aerogenes* as % of total particles
1×10^{9}	10^{-4}	130	0·130
8×10^{8}	10^{-4}	165	0·206
$1·5 \times 10^{9}$	10^{-4}	610	0·406
5×10^{7}	10^{-4}	1310	26·2

Plate 1, fig. 2, is a higher-power electron micrograph of a single μ particle. This shows the outer double membrane very clearly and also shows the lack of differentiation of the internal contents. The granular material in the cytoplasm is poorly defined, and no conclusion can be drawn about its nature. The resemblance to a bacterium is, however, very striking.

Tests for contamination with the food bacterium *Aerobacter aerogenes* A3(o) were

made by dilution and plating of μ-particle suspensions on nutrient agar plates and also by testing the suspensions with antiserum specific against the *A. aerogenes* A 3(o), which is a rough strain of stable serotype. Table 1 shows typical results for the viable bacterial counts of μ particle preparations and preparations from paramecia not bearing μ particles. The results show the approximate upper and lower degrees of contamination. It is evident that, while the number of viable *A. aerogenes* was similar in both preparations, this number as a percentage of the total number of rod-shaped particles was much lower in a μ-particle preparation than in a preparation from paramecia not bearing μ particles, there being at least a 50-fold difference between the former proportion and the latter.

Table 2 shows the results of agglutination tests on the μ particle suspensions with rabbit antiserum prepared against the *Aerobacter aerogenes* A 3(o). It is clear that the μ particles were not at all agglutinated by this antiserum, which had a titre of 1/640 against *A. aerogenes* A 3(o). This result would be expected if the contamination with *A. aerogenes* A 3(o) was as low as that found in the plate counts, and shows that the μ particles were not serologically like the food bacteria.

Table 2. *Contamination of μ preparations with Aerobacter aerogenes A 3(o) agglutination of preparations with anti-A 3(o) serum*

A. Aerogenes A3 (o) was grown up overnight in nutrient broth at 37°, the culture centrifuged, and the deposit washed twice with distilled water. The deposit was taken up in Dryl solution and this suspension adjusted to an extinction scale reading of 1·3 at 650 mμ in a Beckman DB spectrophotometer. The μ suspension, prepared and washed as described, was adjusted to a similar extinction scale reading, also in Dryl solution. Suspension (0·2 ml.) was mixed with 0·2 ml diluted antiserum (Dryl solution as diluent) in agglutination tubes, and incubated overnight in a water bath at 37°. Agglutination was then scored on a scale up to 3+. For the experiments here, the antiserum had a titre of 1/640.

Preparation	Dilution of serum	Agglutination of suspension
μ particles	1/40	-----
μ particles	1/80	-----
μ particles	1/160	-----
μ particles	No serum	-----
A. aerogenes A 3(o)	1/40	+++
A. aerogenes A 3(o)	1/80	+++
A. aerogenes A 3(o)	1/160	++
A. aerogenes A 3(o)	No serum	-----
No bacteria	1/40	-----

Chemical composition of isolated μ particles

Both μ-particle preparations and preparations from paramecia not bearing μ particles were analysed by the methods described. Table 3 gives the results obtained, in terms of μg. component/mg. dry weight of preparation. The yields, in terms of dry weight, were very different for μ-particle preparations and for preparations from sensitive paramecia. The yield of μ particles was about 1 mg. dry weight from 20 to 25 mg. dry weight paramecia; the yield of preparations from paramecia not bearing μ particles was about 1 mg. dry weight from about 500 mg. dry weight of paramecia. Since μ-particle preparations therefore contained about 20 times as much material as preparations from sensitive paramecia, from a similar amount of starting material, this suggests that 95 % of the μ-particle preparations did in fact consist of μ particles, a

degree of purity in agreement with that found from observations by light and electron microscopy. However, the data in Table 3 also suggest that the method of Lowry *et al.* (1951) for the estimation of protein gave an overestimate. It seems possible that this result was due to the presence of some component in the extracts used for the analysis which caused intensification of the colour obtained. All reagents gave blank reactions, and since both *μ*-particle and sensitive-paramecium preparations showed this effect, it seems that it must have arisen from the presence of some contaminating material. It is evident that there were distinct differences between preparations of *μ* particles and preparations from sensitive paramecia, particularly in DNA and RNA content. The *μ* particles contained DNA, RNA, protein and perhaps carbohydrate and lipid.

Table 3. *Chemical composition of isolated μ particles of Paramecium aurelia 540 and of isolated control preparations*

The upper set of figures for both *μ* particles (from *P. aurelia* 540 particle-bearing) and control preparations (from *P. aurelia* 540 sensitive) gives the amount of each component in *μ*g./mg. dry weight, as determined by the methods given. Thus *μ* particles contain 51·4 *μ*g. DNA/mg. dry weight, and so on. The results for *μ* particles are the results of six experiments with separate preparations; the results for control experiments are the means of three experiments with separate preparations. The second line of figures gives the standard errors of the results above them. The third line of figures in the case of *μ* particles is the approximate figures for the composition of 20 mg. dry weight *μ* particles, estimated from the values given above them. The figures for control preparations given in line 4, give an estimate of the contribution of contaminating debris to the typical preparation of *μ* particles, which contained about 20 times as much dry weight of material as a control preparation from paramecia not bearing *μ* particles.

Preparation	DNA	RNA	Protein	Carbohydrate	Lipid
μ particles	51·4	96·0	1035	45·9	11·3
	(±9·1)	(±16·8)	(±242)	(±9·7)	(±2·0)
	(1·028)	(1·92)	(20·70)	(0·918)	(0·226)
Control (540 sensitive)	11·8	53·8	1064	57·5	Not tested
	(±6·1)	(±9·3)	(±162)	(±3·7)	

The DNA of the μ particles

DNA from *Paramecium aurelia* 540 was first characterized by density-gradient centrifugation in CsCl and the determination of the temperature of melting, Tm. For this DNA was prepared from isolated macronuclei of *P. aurelia* 540. This was used for two reasons: (i) it was found that DNA was difficult to purify from whole *P. aurelia*, owing to a high degree of nuclease activity in the extracts, resulting in degraded DNA; (*ii*) the use of DNA prepared from *P. aurelia* macronuclei avoided the possibility of contamination of the DNA with that from any residual *Aerobacter aerogenes*, and resulted in fewer manipulations being necessary to purify the DNA. Macronuclei were isolated as described by Stevenson (1967*c*).

The buoyant density of macronuclear DNA was estimated to be 1·693 g./c.c. (Fig. 1*a*). Densities were estimated by reference to marker DNA of *Micrococcus lysodeikticus* and *Aerobacter aerogenes* A3(0); these have densities of 1·731 g./c.c. and 1·715 g./c.c., respectively (Schildkraut, Marmur & Doty, 1962). A density of 1·693 g./c.c. corresponds to a guanine + cytosine (GC) content of 32 mole%. Heating the DNA solution at 100° for 5 min., then fast-cooling in an ice-bath, resulted in an increase of the buoyant density to 1·706 g./c.c. (Fig. 1*b*). The pattern of change of extinction with temperature was as expected for a double-stranded DNA (Fig. 1*c*) and Tm = 81·8°,

corresponding to GC content of 30·6 mole%. These values are substantially in agreement, and are also in agreement with the values given by Smith-Sonneborn, Green & Marmur (1963), Gibson (1965) and Suyama & Preer (1965).

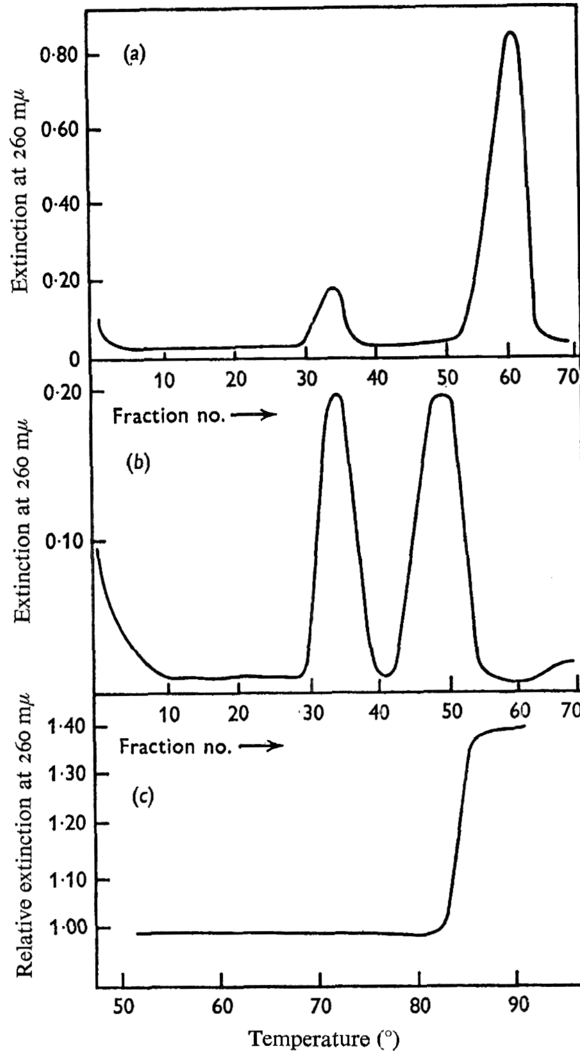

Fig. 1. (*a*) CsCl density gradient of native DNA of *Paramecium aurelia* 540, prepared from isolated macronuclei, with marker of *Micrococcus lysodeikticus* DNA, 210 μg., of *P. aurelia* DNA, 58 μg., of *M. lysodeikticus* DNA, 70 hr at 32,000 rev./min. (100,000 *g*) at 19°. (*b*) As figure (*a*), but 61 μg. heat-denatured DNA of *P. aurelia* 540 substituted for native DNA. (*c*) Melting curve of *P. aurelia* 540 macronuclear DNA. Buffer: SSC. Tm = 81·8°.

Preparation of DNA from μ particles by the Pronase method or by the method of Marmur (1961) was not found feasible, but 2·5 % SDS lysates gave satisfactory results in CsCl gradients. Figure 2 shows the result of density-gradient centrifugation of a μ particle lysate. The peak had a buoyant density estimated to be 1·694 g./c.c. Preparations from paramecia lacking μ particles and treated in the same way showed no

such peak (Fig. 3). Thus μ particles contained DNA capable of forming a peak in a CsCl gradient; preparations from sensitive paramecia did not. To test whether the DNA in the μ-particle preparations did in fact have this density, a mixing experiment in which DNA of macronuclei *Paramecium aurelia* 540 was added to a μ-particle lysate and the mixture subjected to density-gradient centrifugation; Fig. 4*a* shows the results. The use of a lysate did not appear to affect the results obtained; from the size of the peak the two DNA's must have mixed, and must have been very similar in density. Figure 4*a* also shows the results of analyses on gradient fractions by using the diphenylamine method for DNA estimation. It is evident that this method gave similar results to those by the measurement of extinction at 260 mμ.

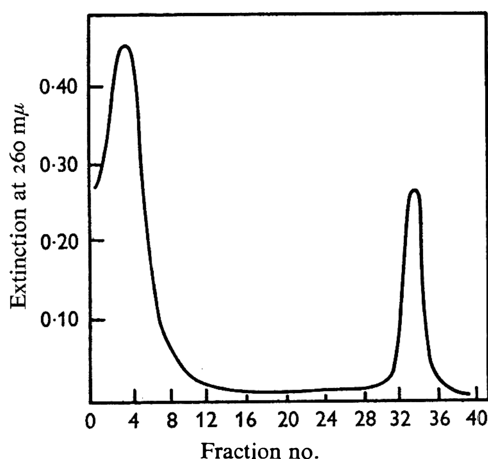

Fig. 2

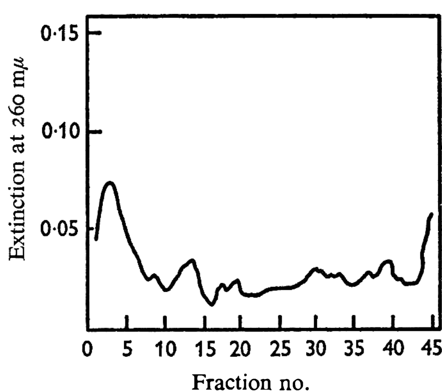

Fig. 3

Fig. 2. CsCl density gradient of 2·5 % SDS lysate of μ particles of *Paramecium aurelia* stock 540. No marker DNA added to this lysate, which contained 91 μg. DNA; 67 hr at 32,000 rev./min. (100,000 × *g*) at 19°.

Fig. 3. CsCl density gradient of 2·5 % SDS lysate of control preparation from *Paramecium aurelia* stock 540 (sensitive paramecia). No marker DNA added; 22 μg. of diphenylamine-reacting material in lysate; 69 hr at 32,000 rev./min. (100,000 × *g*) at 19°.

Figure 4*b* shows the temperature/extinction profile for DNA from μ-particle preparations. The Tm had a mean value, based on several determinations with three different samples, of 82·0°. This led to a value for the GC content of 31·0 mole%, substantially in agreement with the value from density-gradient centrifugation, which was 32·3 mole%.

In further experiments μ particles were treated with deoxyribonuclease (EC 3.1.4.5) before lysis, to test whether the DNA found in μ-particle preparations was outside the particles as a contaminant, or inside and perhaps protected from enzyme action by the wall of the μ particle. Figure 5 shows the result of such an experiment; the legend gives the conditions used. Under these conditions DNA of *Paramecium aurelia* 540 was degraded, and did not give a peak in a CsCl gradient. Since the DNA in μ-particle preparations was not degraded, it seems that it must have been protected from nuclease action, presumably by the outer membrane of the μ particle.

The material banding in CsCl gradients was established as DNA on the basis of the

following criteria: (i) peak material was examined for a typical nucleic acid spectrum, which it was always found to possess; (ii) DNA preparations gave typical temperature/extinction curves, and an increase of buoyant density of about 0·015 g./c.c. occurred on heating to 100° and cooling in ice; (iii) peak material gave a typical reaction with diphenylamine; (iv) the ability to band in CsCl was abolished by treatment with DNase. These are properties which native double-stranded DNA would be expected to possess.

In summary, therefore, the principal findings are that the μ particles possessed DNA, presumably inside the μ particle protected by a membrane. The buoyant density of this DNA, and its Tm, suggest that its base composition is similar to that of the macronuclear DNA of *Paramecium aurelia* 540.

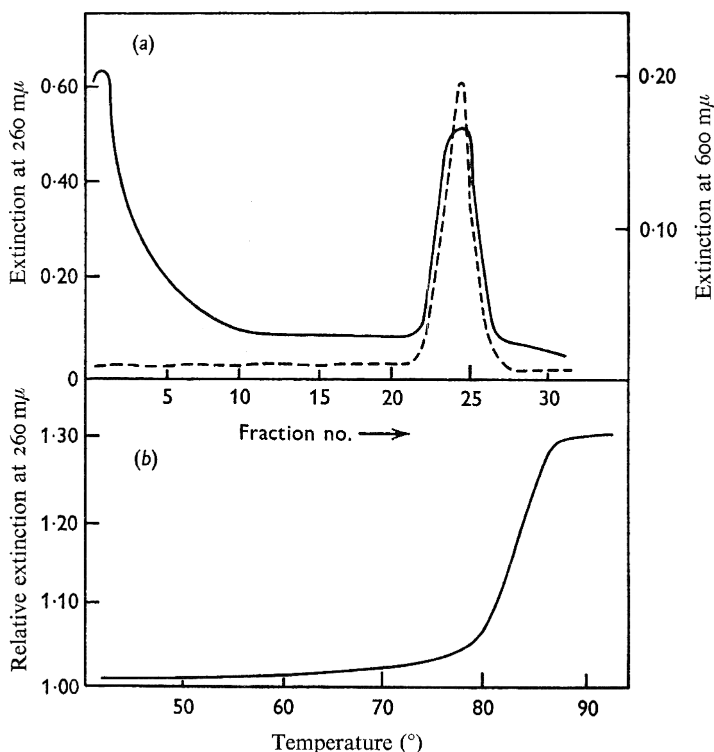

Fig. 4(*a*). CsCl density gradient of μ particles 2·5% SDS lysate+DNA of *Paramecium aurelia* 540 macronuclei. The lysate contained 102 μg. DNA and 79 μg. *P. aurelia* macronuclear DNA. Recovery of DNA in peak = about 135 μg., i.e. more than either component, indicating that the two components have a similar density. ——, Extinction at 260 mμ; ---, extinction at 600 mμ in the diphenylamine reaction; 70 hr at 32,000 rev./min. (100,000 *g*) at 19°. (*b*) Melting curve of DNA from isolated μ particles of *P. aurelia* 540. Buffer: SSC. Tm = 82°.

Ribonucleoside triphosphate incorporation by μ particles of Paramecium aurelia 540

Experiments were made to test whether the μ particles possessed a mechanism for RNA synthesis, by assaying the incorporation by μ particles of radioactive nucleoside triphosphates into acid-insoluble material. Experiments were also made with control preparations from paramecia not bearing μ particles.

Table 4 gives typical results on the incorporation of ^{14}C-ATP and ^{3}H-GTP into acid-insoluble material by isolated μ particles lysed by three cycles of freezing and thawing, and on the incorporation of ^{14}C-ATP by similarly treated preparations from sensitive paramecia. All results are pmoles of labelled triphosphate incorporated into acid-insoluble material in 15 min. at 30°. The various additions to and omissions from the assay mixture are detailed in the legend to Table 4. Incorporation of UTP was very similar to that of ATP (561·3 pmoles with the complete mix), but these results are not presented in detail because only one series of experiments with this compound was made. All other experiments had at least four replications. Table 4 shows that the μ particles had a very active nucleoside triphosphate incorporating activity. Preparations from sensitive paramecia always gave negative results, there being no incorporation in any of seven replications of this experiment. This suggests that the incorporation was indeed due to the μ particles.

Table 4. *Ribonucleoside triphosphate incorporation by isolated μ particles and control preparations from Paramecium aurelia 540 and 540 (sensitive)*

All figures are $\mu\mu$mole triphosphate incorporated into acid-insoluble material in 15 min. at 30°/mg. protein in the experimental extract.

	μ particles				Control
Additions/omissions to assay mixture	^{14}C-ATP pmole	Inhibition (%)	^{3}H-GTP- $\mu\mu$mole	Inhibition (%)	^{14}C-ATP pmole
None (complete system)	595·7	0·0	367·5	0·0	0
No ATP	—	—	72·9	80·2	—
No GTP	77·8	86·9	—	—	0
No CTP	230·0	61·4	31·9	91·3	0
No UTP	287·1	51·8	109·6	70·2	0
No triphosphates	180·2	69·7	81·9	77·7	0
+ Actinomycin D*	260·2	56·3	59·0	83·9	0
+ Ribonuclease†	42·3	92·9	—	—	0
No Mg^{2+}	0·0	100	—	—	0

* Concentration of Actinomycin D was 15 μg./ml. Actinomycin D was a gift from Merck, Sharp and Dohme, Inc., Rahway, N.J., U.S.A.

† Concentration of ribonuclease was 25 μg./ml.

Control preparations gave negative results in every experiment (seven replicates).

For optimal activity the system required all four triphosphates and the presence of Mg^{2+} ions, the optimal concentration of these being 5 mM. The system was susceptible to actinomycin D, known to inhibit DNA-dependent RNA synthesis, and an RNAase-sensitive product was synthesized. The optimum pH value was not sharp, and lay in the range pH 8·1 to 9·0; incorporation decreased sharply at pH values outside this range. This range was found by Weiss (1960; 1962) to be optimal for DNA-dependent RNA synthesis. The findings thus suggest that RNA is synthesized in a DNA-dependent reaction by the μ particles.

Figure 6 shows the time-course of incorporation of ^{14}C-ATP. Incorporation normally ceased after 15 to 20 min., but on occasion continued to 25 to 30 min. ^{14}C-UTP and ^{3}H-GTP gave similar time-courses. Loss of TCA-insoluble label occurred to 60 min.; after this time over half the label incorporated in 15 min. had been lost. That there was no increase of TCA-insoluble label on prolonging the incubation suggests that there was little bacterial contamination, but that degradative processes were occurring.

From experiment to experiment variation in the amount of inhibition was observed when one or more nucleoside triphosphates was absent from the assay mixture; this may have reflected variations in the amount of nucleoside triphosphate present in the μ particles, or there may have been differences in the isolation procedures which caused this. This variation was not more than $\pm 15\%$. Activity was retained after storage up to 1 month in the frozen state (longer periods not tested). When a μ-particle preparation was more contaminated with cell debris than usual, the preparation had a markedly lower incorporating activity.

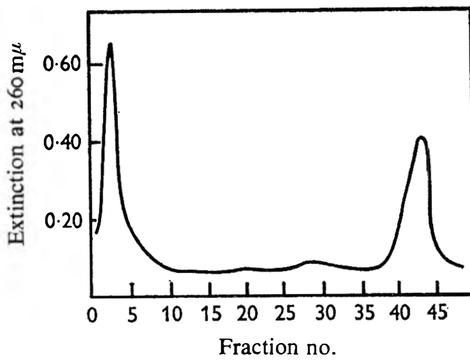

Fig. 5

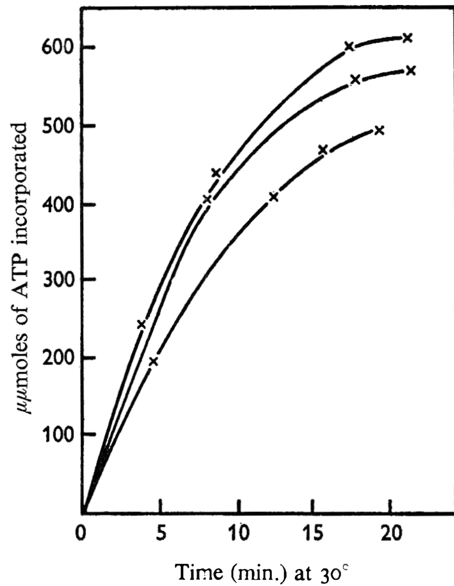

Fig. 6

Fig. 5. CsCl density gradient of 2·5 % SDS lysate of μ particles of *P. aurelia* 540 which was treated with DNase 10 μg./ml. before lysis. DNase treatment was for 45 min. at 37°; no marker DNA was added after lysis; the lysate contained 38 μg. DNA; 68 hr at 32,000 rev./min. (100,000 $\times g$) at 18°.

Fig. 6. Time-course of incorporation of 8-^{14}C-ATP by isolated μ particles of *P. aurelia* 540. Conditions as described in text. Three experiments with separate preparations are shown.

DISCUSSION

From the results presented in this paper it seems more firmly established that the μ particle is indeed a symbiotic bacterium. It resembles a bacterium in size, shape and appearance in the electron microscope. The chemical composition, the presence of DNA and RNA, and the ability to synthesize RNA are compatible with this hypothesis. In their appearance in the electron microscope, μ particles resemble Gram-negative bacteria. Nuclear bodies, as observed in many species, are not evident in the isolated μ particle. Beale & Jurand (1960) concluded that the DNA was spread throughout the interior of the particle and also stated (Beale & Jurand, 1966) that fine filaments resembling DNA may be observed in the interior of the particle. This is not evident in Pl. 1, fig. 2, but there may be changes during isolation which might account for this. If the DNA of the particle was not localized then it would not show up as a distinct

region. Sonneborn (1959) suggested that this might also be the case for the kappa particle.

The cell wall of the μ particle shows great resemblance in fine structure to the cell walls of other bacteria. The resemblance is closest to a Gram-negative bacterium, and it is of interest that μ particles have a negative Gram-reaction, and they are not acid-fast. It has been shown (Stevenson, 1967*a*) that the μ particle contains α,ϵ-diaminopimelic acid, presumably as a constituent of the cell wall.

As an endosymbiotic bacterium, the μ particle must have at some time invaded the Paramecium cytoplasm, presumably via the buccal cavity. There is no evidence that the comparatively rigid and highly complex cell wall of Paramecium is capable of undergoing any process comparable to pinocytosis, though this process occurs in the food vacuoles. There seems little chance of infection through the cytopyge, as the opening there is small and short-lived at the discharge of old food vacuoles, and any flow is outward. Jurand (1961) showed this in an electron microscope study, and also observed that breakage of a food vacuole before discharge must be a rare event. This makes it difficult to postulate an infection route. But Beale & Jurand (1966) observed that many wild stocks of *Paramecium aurelia* contain endosymbiotes, though these are frequently lost on culture in the laboratory. This would suggest that endosymbiosis is a flexible condition, established and lost with change of the cell's environment, which suggests that infection must be common. Infection of cells with symbiotes has been achieved with several particles, but not with the μ particle, though this may be because the correct conditions have not been found.

The mate-killing effect of the μ particle is in itself rather perplexing, as it results in the death of half the exconjugant animals from any one cross. Siegel (1954) showed that in syngen 8 mate-killers, physical contact of the mate-killer and sensitive animals was necessary, and that the main killing action was due to interference with the prezygotic macronucleus, possibly at the level of nucleic acid metabolism. Gibson & Beale (1962) showed that in stock 540 complete conjugation with the exchange of genes was necessary for killing action. However, a fruitful approach to this problem is difficult to devise.

The results presented in this paper have shown that the μ particle of *Paramecium aurelia* stock 540 possesses some of the attributes of an organism. It possesses DNA, is able to direct the synthesis of RNA, shows comparable ultrastructure to a free-living bacterium; in fact has characteristics of a free-living cell. But so far it has only been found to have a habitat in the Paramecium cytoplasm. In this respect, therefore, it is comparable to a cell organelle. However, where the μ particle differs from organelles such as the chloroplast and mitochondrion is that it lacks their specialization of function, for since the Paramecium can survive and develop normally without the μ particle, it cannot have any essential function. However, it may be able to carry out synthetic functions that the host cell cannot, as the lambda particle of stock 299 (syngen 4) is able to do (Soldo, 1963). Kung (1966) has shown that the kappa particle of stock 51 can respire and utilize glucose *in vitro*. Thus it would seem that all these particles possess considerable metabolic ability. This would seem very likely if the endosymbiotic particles of Paramecium are indeed taken up and lost with comparative frequency, for then the endosymbiotes would be bacteria which could live in the same habitat as Paramecium in the wild. It seems simplest, therefore, to regard these particles as bacteria capable of living endosymbiotically and possibly giving benefit to the host

cell, which in at least some cases has evolved mechanisms in its genes (in the case of stock 540, the M genes) for assisting in the maintenance of this state. Clearly, this process may be regarded as a stage toward an obligate intracellular existence for the particle, a stage in the evolution of an organelle carrying out functions indispensable to the host cell.

I should like to thank Professor G. H. Beale, F.R.S., for his frequent advice and criticism, Mr R. Sinden for carrying out the electron microscopy, Miss A. Wightman for help with the cultivation of the paramecia, and others who have offered suggestions, advice and criticism.

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Fig. 1

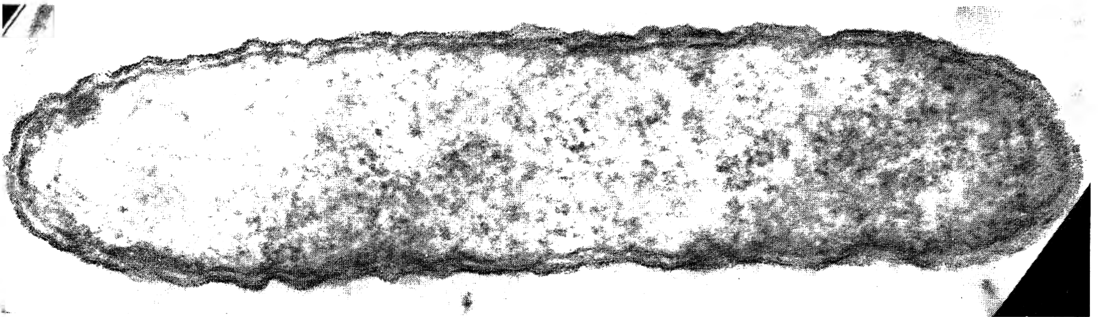

Fig. 2

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EXPLANATION OF PLATE

PLATE 1

Fig. 1. Low-power electron micrograph of a preparation of *μ* particles. Specimen preparation as detailed in the text. Shows *μ* particles in various stages of preservation—some are undergoing lysis. ×7500.

Fig. 2. Higher-power electron micrograph of a single *μ* particle. Preparation as detailed in the text. The outer double membrane is clearly shown, as is the ill-defined appearance of the internal contents of the particle. ×40,000.

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The Cause of Loss of Viability of Airborne *Escherichia coli* K12

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SUMMARY

The uptake of uracil, pyruvate, methionine and oxygen and the breakdown of RNA and protein have been examined in *Escherichia coli* K12 HfrC after storage as an aerosol for 15 min. in nitrogen at 80 % and 40 % relative humidity (RH). At 80 % RH there was greatest loss of viability and almost complete breakdown of RNA together with severe inhibition of energy production. At 40 % RH all these effects were mitigated.

INTRODUCTION

Microbial survival has been reviewed by Anderson & Cox (1967). The aerosol survival of *Escherichia coli* K12 has been discussed in detail by Cox (1968*b*). At high RH values in atmospheres devoid of oxygen, critical minima occur in the survival versus RH curves (Cox, 1966). These minima may be caused by failure of RNA synthesis, of protein synthesis or of energy production (Cox, 1968*b*). This paper describes further work on the cause of death in *E. coli* K12 HfrC aerosols.

METHODS

Growth of organisms. Escherichia coli K12 HfrC was grown in a 2 % tryptone medium (pH 7·2) for 16 hr at 37° in a shaken flask. The bacteria were centrifuged and resuspended in glass-distilled water to 10^{11} bacteria/ml. before spraying.

Apparatus. The aerosol was produced by means of a 3-jet collision spray (Collison, 1935) operated with nitrogen (> 99·9 %). It was mixed with more nitrogen of controlled temperature (26·5° ± 0·2) and RH, and flowed into a 75 l. rotating drum (Goldberg, Watkins, Boerke & Chatigny, 1958). The 3-jet spray was used because high densities of bacteria were required in some of the experiments. Control of RH was not as good as with the 1-jet spray (Cox, 1968*a*) but neither was it so critical with the present organism (Cox, 1968*b*). Aerosol samples after storage for 15 min. were collected in a Porton 'raised' impinger (May & Harper, 1957) containing 10 ml. of collecting fluid, and the bacterial concentration estimated optically. Phosphate buffer (Cox, 1966) or minimal growth medium were collecting fluids. The minimal growth medium contained 1·0 % (w/v) glycerol; 0·5 % ammonium citrate; 1·0 % $K_2HPO_4.3H_2O$; 0·05 % $MgSO_4.7H_2O$; 0·05 % NaCl; 0·005 % ferric ammonium citrate adjusted to pH 7·0; with NaOH.

Uptake of labelled substrates. Suspensions (2·5 × 10^8 bacteria/ml.) either collected from the aerosol or made up from non-aerosolized controls, both in minimal growth

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medium, were incubated at 37° in separate shaken flasks containing 0·025 μc/ml. of one of the following substrates: [^{14}C]uracil (for labelling RNA), Na[^{14}C]pyruvate (for determining the general metabolic rate of the bacterial cell), [^{14}C]thymidine (for labelling DNA) or [^{35}S]methionine (for labelling protein) (The Radiochemical Centre, Amersham, Bucks). Samples taken at intervals were filtered through a Millipore filter, which was subsequently well washed with ice-cold 5 % trichloracetic acid; the filtrates combined with the washings were assayed for radioactivity at room temperature by a coincidence scintillation technique (Anderson & Smith, 1965). The amount of each substrate incorporated into the bacteria was then estimated by the decrease in the amount of radioactivity in the corresponding filtrate (Benbough, 1967).

Breakdown of RNA or protein. Bacteria containing labelled RNA or protein were grown by adding 0·05 MC of ^{14}C-uracil or 0·1 MC. of ^{35}S-methionine to 100 ml. of the tryptone medium at the time of inoculation with *Escherichia coli* K12 HfrC. After aerosolization and collection into minimal growth medium, the amount of labelled uracil or methionine released by the bacteria on incubation was followed by a procedure similar to that detailed above.

Oxygen uptake. The aerosol was collected into phosphate buffer (Cox, 1966) to give 5×10^8 bacteria/ml. Oxygen uptake at 37° was determined by the Warburg technique with tryptone medium in the flask, bacteria in the side-arm and concentrated sodium hydroxide in the centre-well, and compared with the uptake from non-aerosolized bacteria.

RESULTS

RNA metabolism. Survival was zero after 15 min. in aerosol at 80 % RH and a small initial uptake of uracil was followed by zero uptake; at 40 % RH the survival was 18 % and there was a much greater uptake of uracil (Fig. 1). Hence at 80 % RH RNA synthesis was drastically reduced, while, in accordance with the levels of survival, at 40 % RH RNA synthesis was greater. At 80 % RH considerable breakdown of prelabelled RNA occurred (Fig. 2); at 40 % RH, where survival was higher, the amount of RNA breakdown was less.

Protein metabolism. Figure 3 indicates that the uptake of pyruvate was only slightly inhibited by aerosolization at 40 % RH, while at 80 % RH the uptake was less, but still quite appreciable. The uptake of methionine was considerably inhibited at both RHs (Fig. 4). At 80 % RH release of [^{35}S]labelled protein was slight while at 40 % RH

Fig. 1. [^{14}C]uracil uptake by *Escherichia coli* K12 HfrC. ×, Control unaerosolized bacteria; ⊙, bacteria stored in nitrogen for 15 min. at 80 % relative humidity; ⊡, bacteria stored in nitrogen for 15 min. at 40 % relative humidity.

Fig. 2. Breakdown of prelabelled [^{14}C]RNA by *Escherichia coli* K12 Hf C. ×, Control unaerosolized bacteria; ⊙, bacteria stored in nitrogen for 15 min. at 80% relative humidity; ⊡, bacteria stored in nitrogen for 15 min. at 40% relative humidity.

Fig. 3. Sodium [^{14}C]pyruvate uptake by *Escherichia coli* K12 HfrC. ×, Control unaerosolized bacteria; ⊙, bacteria stored in nitrogen for 15 min. at 80% relative humidity; ⊡, bacteria stored in nitrogen for 15 min. at 40% relative humidity.

Fig. 4. [^{35}S]methionine uptake by *Escherichia coli* K12 HfrC. ×, Control unaerosolized bacteria; ⊙, bacteria stored in nitrogen for 15 min. at 80% relative humidity; ⊡, bacteria stored in nitrogen for 15 min. at 40% relative humidity.

Fig. 5. Oxygen uptake by *Escherichia coli* K12 HfrC. ×, Control unaerosolized bacteria; ⊙, bacteria stored in nitrogen for 15 min. at 80% relative humidity; ⊡, bacteria stored in nitrogen for 15 min. at 40% relative humidity.

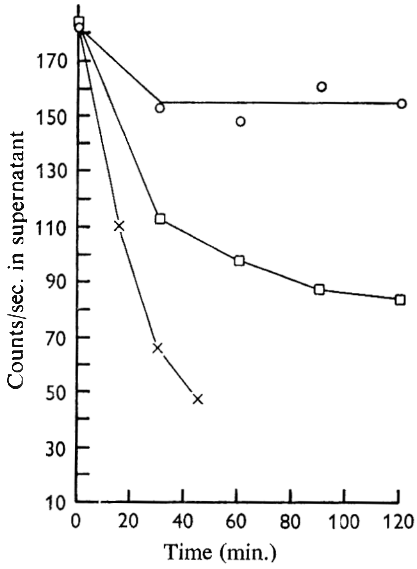

Fig. 1

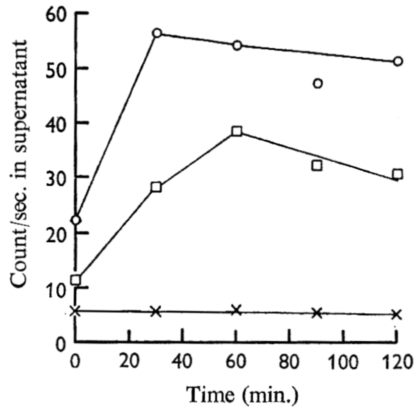

Fig. 2

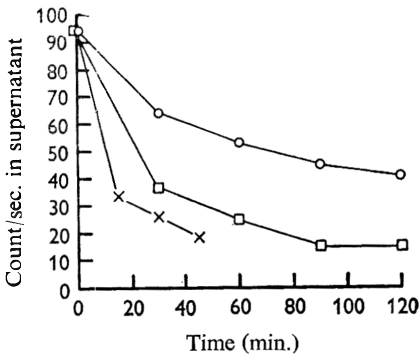

Fig. 3

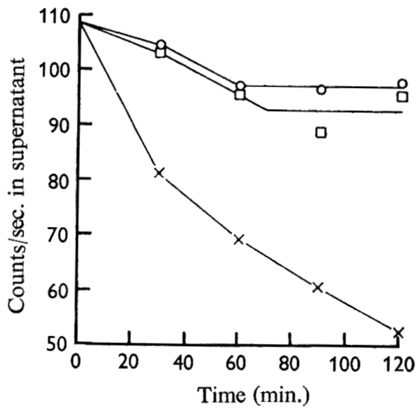

Fig. 4

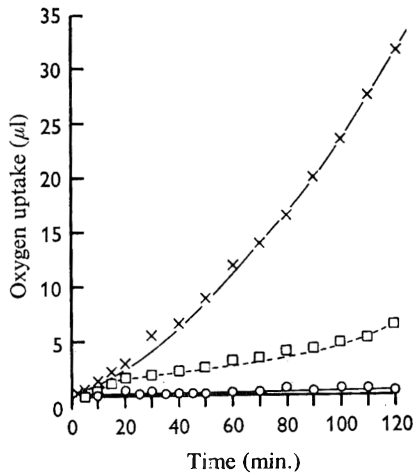

Fig. 5

it was extremely small and very comparable to that from an unsprayed control. Loss of viability therefore did not arise from loss of protein.

Oxygen uptake. A severe inhibition of oxygen uptake occurred for bacteria collected from the aerosol at 80 % RH, while at 40 % RH the degree of inhibition was less, but still significant (Fig. 5).

DISCUSSION

Aerosol loss of viability was greatest in the region of 80 % RH and least in the region of 40 % RH (Cox, 1968*b*) and these values were therefore chosen for the present studies.

Most of the RNA, as measured by the release of radioactivity, was broken down following aerosolization at 80 % RH. The failure of the bacteria to take up oxygen following recovery from the aerosol meant that no energy was available for resynthesis of RNA, and inevitably led to death of the organism. At 40 % RH RNA breakdown was incomplete, and oxygen uptake, although sub-normal, was still evident.

An unstressed bacterium contains RNA complexed with protein and also ribonuclease, which normally does not break down the ribosomal RNA. However, if in the aerosol the RNA-protein complex dissociates then the RNA can be attacked and broken down by the enzyme.

Water movement in the region of 80 % RH might cause a dissociation of the RNA-protein complex; Rich & Watson (1954) have suggested that RNA undergoes structural changes depending upon RH. Previous evidence reviewed by Cox (1968*b*) pointed to damage on rehydration following collection from the aerosol; on this basis rehydration caused dissociation of the RNA-protein complex, with the other changes following. The higher survival at 40 % RH is only to be expected considering the all-round improvement in metabolism; the main puzzle is why rehydration from 40 % RH should be so much less damaging than rehydration from 80 % RH.

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The Cultivation of the Rumen Ciliate *Entodinium simplex*

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SUMMARY

Entodinium simplex has been maintained *in vitro* anaerobically and in the presence of bacteria in a salts medium containing autoclaved rumen fluid, wholemeal flour and dried grass. The protozoa divided at least every 3 days during more than $2\frac{1}{2}$ years. The wholemeal flour could be replaced by a mixture of rice starch and washed bran but not by either separately; in the presence of this mixture population densities of over 60,000 protozoa/ml. were obtained. Omission of the autoclaved rumen fluid decreased the number of protozoa to 25 % of this value. Evidence was obtained that it was the particulate matter in the rumen fluid that was an essential for growth.

INTRODUCTION

Over the last 25 years rumen Entodiniomorphid protozoa have been grown successfully *in vitro* several times, but the population densities reported have usually been low. Hungate (1942, 1943) maintained several diplodinia for over a year at population densities of less than 1000/ml. Gutierrez & Davis (1962) cultivated *Epidinium ecaudatum in vitro* for 5 months but obtained populations of only 1200/ml. Clarke (1963) maintained *Eremoplastron bovis* in unspecified numbers for 5 months and mixed entodiniomorphs for somewhat longer, while Mah (1964) maintained up to 1000 *Ophryoscolex purkynei*/ml. for nearly 3 years with a mean generation time of 24 hr. The longest period of continuous cultivation of a single species has been 9 years (1959 to the time of writing) for *Entodinium caudatum*, by the present author. This protozoon has been maintained at a population density of 30,000/ml. and divided every 2 to 3 days (Coleman, 1960).

Recently Jarvis & Hungate (1968) maintained *Entodinium simplex*, dividing every 24 hr, for an unspecified time on a medium of ground wheat and grass. Although these authors reported protozoal counts up to 30,000/ml. in the presence of certain additives, many of their cultures declined rapidly and inexplicably from these peak numbers, and steady-state population densities of only 8000/ml. were obtained.

The present paper describes the successful cultivation of *Entodinium simplex* (Dogiel) for over 2 years in a buffered salts medium with wholemeal flour and dried grass as the sources of carbohydrate; a preliminary communication has already appeared (Coleman, 1968). Although the name *Entodinium simplex* is widely used and will be used in this paper, some authors, e.g. Zielyk (1961) and Lubinsky (1957), consider that this protozoon is the same as *Entodinium dubardi dubardi* (Buisson) and that this latter name, being the earlier, is the correct one.

METHODS

Culture media. The standard medium consisted of 20 ml. autoclaved (115° for 20 min.) mineral salt solution; 4 ml. 5 % $NaHCO_3$ (freshly prepared in autoclaved water); 0·6 ml. 1 % L-cysteine HCl (neutralized immediately before use); 1·0 ml. aqueous 1·5 % suspension of 'stone ground' wholemeal flour (W. Prewett Ltd., Stone Flour Mills, Horsham, Sussex); 3 ml. autoclaved rumen fluid (ARF—see below); 5 mg. dried grass (Coleman, 1960); contained in a 50 ml. centrifuge tube; CO_2 was then bubbled vigorously through the complete medium for 3 min. and the tubes stoppered immediately with a rubber bung. The mineral salt solution contained (g./100 ml.): K_2HPO_4, 0·49; KH_2PO_4, 0·38; NaCl, 0·127; $CaCl_2$ (dried), 0·0035; $MgSO_4.7H_2O$, 0·007.

Sterile rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay and oats. After treatment of fresh rumen contents as described below the fractions were autoclaved (115° for 15 min.) under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in sealed McCartney bottles. The fresh rumen fluid was first strained through two layers of muslin and when autoclaved formed protozoa-containing autoclaved rumen fluid (PARF). This strained fluid was then centrifuged at 500 *g* for 3 min. and the supernatant fluid when autoclaved formed ARF. This supernatant fluid was further centrifuged at 10,000 *g* for 20 min. and the supernatant fluid from this centrifugation autoclaved to form autoclaved supernatant fluid (AS). The pellet from this second centrifugation was washed once in mineral salt solution (Coleman, 1958) and then, after suspension in the original volume of salt solution, was autoclaved to form autoclaved bacteria (AB).

Source of protozoa. The protozoa for inoculation were prepared from the rumen contents of Clun Forest sheep fed on hay and oats. The rumen fluid was taken 6 hr after feeding and strained through one layer of muslin to remove the larger food particles. The fluid was then allowed to stand in an 8 × 1 inch (20 × 2·5 cm.) tube at 39° for 2 hr to allow the larger protozoa to fall to the bottom of the tube. The fluid from the top of the tube was then centrifuged at 600 *g* for about 3 min. or until one drop of the supernatant fluid showed on microscopic examination *Entodinium simplex* as the only ciliate protozoon present.

Cultural conditions. The cultures were first established in 15 ml. centrifuge tubes containing only 10 ml. of standard medium and to which was added only 0·1 ml. of 1·5 % aqueous suspension of wholemeal flour. These tubes were inoculated with 1 to 5 drops of the centrifuged rumen contents containing *Entodinium simplex*, gassed with CO_2, sealed with a rubber bung and incubated at 39°. Wholemeal flour (0·1 ml. of 1·5 % aqueous suspension) and about 1 mg. dried grass was added to each tube every day and after 10 to 14 days the medium was examined microscopically for the presence of *E. simplex*. In most experiments 50 to 100 protozoa/ml. were present after this time, but when none was visible in a drop of culture the incubation was continued for a further week. If there were still no protozoa present the culture was discarded, as were any tubes that were found at any stage to contain caudate protozoa. As it was easy inadvertently to transfer protozoa from one tube to another by interchanging rubber bungs or by droplets of culture on the end of the pipette used to gas the tubes, care was taken to ensure that each tube always had the same bung and that the pipette was flamed each time before use. When these precautions were not taken, a more

vigorously growing contaminant protozoon, such as *E. caudatum*, could be rapidly transferred from one tube to all the others in a batch.

Two or three weeks after the initial inoculations the cultures were diluted with an equal volume of fresh medium containing 0·2 ml. aqueous 1·5 % suspension of wholemeal flour, and transferred to a 50 ml. centrifuge tube. The quantity of 1·5 % wholemeal flour suspension added daily was gradually increased to 0·5 ml. and then doubled to 1·0 ml. The cultures were diluted with an equal volume of fresh medium once a week for a month, after which time this was increased to twice each week. Stock cultures were maintained on the standard medium with the daily addition of 1·0 ml. 1·5 % suspension of wholemeal flour and 2 mg. dried grass and were diluted with an equal volume of fresh medium twice a week, i.e. condition D 3–4 of Coleman (1960). The fresh medium contained twice the amount of wholemeal flour normally added, in order to maintain the final concentration constant.

Protozoal counts. The number of protozoa in a culture was estimated by blowing 0·5 ml. of the culture from a 1 ml. graduated pipette with a large hole at the tip into 5 ml. 0·02 M-iodine and counting microscopically all the protozoa in 0·1 ml. of the mixture. Only those protozoa which showed no signs of disintegration were counted. Where the effect of a change in growth medium is reported, at least 3 weeks had been allowed to elapse before the number of protozoa present was estimated. Unless otherwise stated the number of protozoa in a culture was always estimated immediately before dilution of that culture.

Preparation of washed bran. Twenty g. of broad bran (H. F. & E. Ison Ltd., Henison Granaries, Histon Rd., Cambridge) were placed in a 500 ml. measuring cylinder and shaken with 500 ml. tap water. The bran was sallowed to settle and the water removed by decantation. This washing procedure was repeated five more times or until the supernatant fluid was clear. The bran was then washed a further four times in distilled water, spread on a flat dish and allowed to dry at room temperature. When the bran was dry it was ground in a Lee Attrition Mill (Lee Engineering Co., Milwaukee, Wis., U.S.A.).

RESULTS

For the continued maintenance of *Entodinium simplex* in the presence of bacteria it was necessary to add fresh wholemeal flour to the protozoal cultures each day. When no such addition was made all the protozoa died within 40 hr of the omission. At no stage after the initiation of the culture was it found necessary to replace the medium completely each day; stock cultures remained alive up to 3 weeks without the addition of fresh medium provided that wholemeal flour was added each day. When *E. caudatum* was grown in the presence of autoclaved rumen fluid it was necessary to replace the medium before dilution of the culture with an equal volume of fresh medium, i.e. condition C 3–4 of Coleman (1960). Fortunately it was possible to maintain healthy cultures of *E. simplex* by direct dilution with an equal volume of fresh medium twice each week. Attempts to decrease the mean generation time of the protozoa by dilution of the culture every 24 or 48 hr instead of twice a week were only partially successful. After 3 weeks there were only 1100 protozoa/ml. when the cultures were diluted every day, as compared with 21,400/ml. when the cultures were diluted every other day, and 26,900/ml. under standard conditions when dilution was twice a week. The actual population densities obtained in individual experiments under standard

conditions were variable and in ten experiments with a mean of 27,000/ml. the range was 16,500 to 61,000/ml. Although the maximum yield of protozoa, in terms of organisms present per protozoon present initially after a given period, was usually greatest when the cultures were diluted every other day, whole cultures occasionally died inexplicably under these conditions and it was safer to maintain stock cultures by dilution twice each week.

The organisms

The protozoa initially selected for the inoculation were the smallest ($44 \mu \times 26 \mu$) non-caudate ciliate protozoa present in the rumen of sheep fed on hay and oats. Since at no time during the $2\frac{1}{2}$ years that this culture has been maintained have any caudate protozoa been observed microscopically, the morphological form of the ciliates cannot be the result of the loss of a caudal spine as has been observed with some rumen protozoa *in vitro* (Coleman, 1963; Clarke, 1963). In stock cultures the size of the ciliates was $40 \mu \times 25 \mu$ (range 35 to $50 \mu \times 23$ to 28μ) which is very similar to that recorded for *Entodinium simplex* by Dogiel (1927).

Effects of medium constituents

The detailed effects of varying in turn each constituent of the standard medium and of adding other materials were as follows.

Wholemeal flour. The daily addition of wholemeal flour to a final concentration of 0·25, 0·5 or 1·0 mg./ml. resulted in protozoal populations of 12,500, 20,600 and 22,400 ciliates/ml., respectively. In the presence of the highest concentration of wholemeal flour the results were variable although the above result was typical of that obtained in about half the experiments made. However in some experiments fewer protozoa were present than when only 0·5 mg. wholemeal flour/ml. was added daily, whereas in others up to 50,000/ml. were present. Replacement of the wholemeal flour in the standard medium by an equal quantity of rice starch decreased the population to 3,700/ml. and replacement by maize, potato or wheat starch resulted in the death of the culture in 4 weeks. When the amount of rice starch added daily was decreased by half to 0·25 mg./ml. the number of protozoa increased to 6100/ml., but at no concentration was a population density of over 7000/ml. ever obtained. This poor growth on rice starch differentiates *Entodinium simplex* from *E. caudatum* which grew readily on both rice starch and wholemeal flour (Coleman, 1958, 1960).

Although most of the starch grains in wholemeal flour were larger than those in commercial rice starch, some were smaller. It was therefore possible that *Entodinium simplex* was living on the small grains in wholemeal flour but dying in the presence of rice starch and other starches because the grains were too large for it to engulf. This hypothesis was supported by the observations of Sugden (1953), who stated that *E. simplex* was unable to engulf rice starch grains and inferred that they were too large to be taken up. Unfortunately it has not been possible to substantiate this and *E. simplex* organisms in crude rumen fluid and in culture have been observed microscopically to engulf rice starch grains and the smaller grains in wholemeal flour. Some protozoa contained starch grains the diameter of which was nearly half that of the engulfing ciliate.

Bran and rice starch. Since one of the products of milling whole wheat to obtain white flour is bran, its effect on protozoal growth in the presence of rice or wheat

starches was investigated. The bran was either used as commercially available, when it contained an appreciable amount of starch, or after washing with water to remove this starch. When ordinary bran was used as a replacement for wholemeal flour at approximately 0·5 mg./ml. the number of protozoa decreased from 26,900 to 3900/ml. but this bran had a slight stimulatory effect when added in addition to wholemeal flour (Table 1). Washed bran supported only 1100 protozoa/ml. However, when ordinary or washed bran was added in addition to rice starch the number of protozoa increased to approximately the population density obtained in the presence of wholemeal flour (Table 1). However, with continued culture in the presence of rice starch (0·25 mg./ml.

Table 1. *Effect of various starches, bran and sterile rumen fluid fractions on the maintenance of Entodinium simplex*

The experiment was made anaerobically in 50 ml. centrifuge tubes containing 20 ml. salts medium, 4 ml. 5% $NaHCO_3$, 0·6 ml. 1% L-cysteine hydrochloride and the additions shown below, equilibrated with CO_2. The cultures were diluted with an equal volume of fresh medium twice each week and fresh flour, starch or washed bran as indicated was added each day. The experiment was initiated with a culture grown on the standard medium and the number of protozoa were counted after maintenance for 3 weeks on the medium shown.

Daily addition of carbohydrate (mg./ml. medium)	Rumen fluid fraction added (10%, v/v)	No. of protozoa/ ml. culture
A		
0·5 wholemeal flour	Autoclaved rumen fluid (ARF)	26,900
0·5 wholemeal flour, 0·6 bran	Autoclaved rumen fluid (ARF)	36,600
0·5 wholemeal flour, 0·6 washed bran	Autoclaved rumen fluid (ARF)	36,300
0·25 rice starch	Autoclaved rumen fluid (ARF)	6,100
0·25 rice starch, 0·6 washed bran	Autoclaved rumen fluid (ARF)	30,400
0·25 wheat starch	Autoclaved rumen fluid (ARF)	600
0·25 wheat starch, 0·6 washed bran	Autoclaved rumen fluid (ARF)	8,400
0·6 bran	Autoclaved rumen fluid (ARF)	3,900
0·6 washed bran	Autoclaved rumen fluid (ARF)	1,100
B		
0·5 wholemeal flour	None	3,700
	AS*	16,700
	PARF†	25,000
	ARF‡	29,400
	AB§	33,000

* AS = rumen fluid centrifuged 10,000 *g* supernatant, autoclaved.
† PARF = whole rumen fluid, autoclaved.
‡ ARF = lightly centrifuged rumen fluid, autoclaved.
§ AB = 10,000 *g* centrifuged pellet from AS, autoclaved = autoclaved bacteria.

daily) and washed bran (0·6 mg./ml. daily) the population density increased until, at the time of writing, cultures of *Entodinium simplex* had been maintained at population densities of over 60,000/ml. for more than 6 months. The number of ciliates was dependent on the amount of washed bran and rice starch added daily up to at least 0·6 mg. of each/ml. (Fig. 1). Unfortunately, at the higher concentrations of rice starch the population densities were liable to decrease to low values inexplicably, and to obtain stable densities the daily addition of only 0·25 mg./ml. was used.

To determine whether the essential factors in the bran persisted in the medium for more than 24 hr the following experiment was made. In the presence of 0·25 mg. rice starch/ml. daily, the further addition daily of 0·6 mg. washed bran/ml. increased the number of protozoa from 6000 to 61,000/ml. When the washed bran was added only

every other day or twice a week the population density decreased to 36,300 and 30,800/ml., respectively. Addition of twice the amount (i.e. 1·2 mg./ml.) of washed bran every other day increased the number to only 40,200/ml., suggesting that some essential factor in the washed bran was only available when it was added each day. When the washed bran was autoclaved (115° for 20 min.) and then added daily at 0·5 to 1·0 mg./ml. the population density was about 36,000/ml., showing that one or more of the essential factors in the bran was sensitive to heat. The washed bran was not replaceable by the following proteins: 0·3 mg. alpha protein/ml., 1·2 mg. zein/ml. or 1·2 mg. casein/ml. However, in the presence of 0·3 mg. wheat gluten/ml. daily the steady-state population density was 70 % of that in the presence of 0·6 mg. washed bran; but gluten had no further stimulatory effect when added in addition to washed bran. It is considered likely that the gluten preparation contained the same stimulatory factor as the washed bran, since they were both prepared from whole wheat and the proteins *per se* had no effect on the growth of the ciliates.

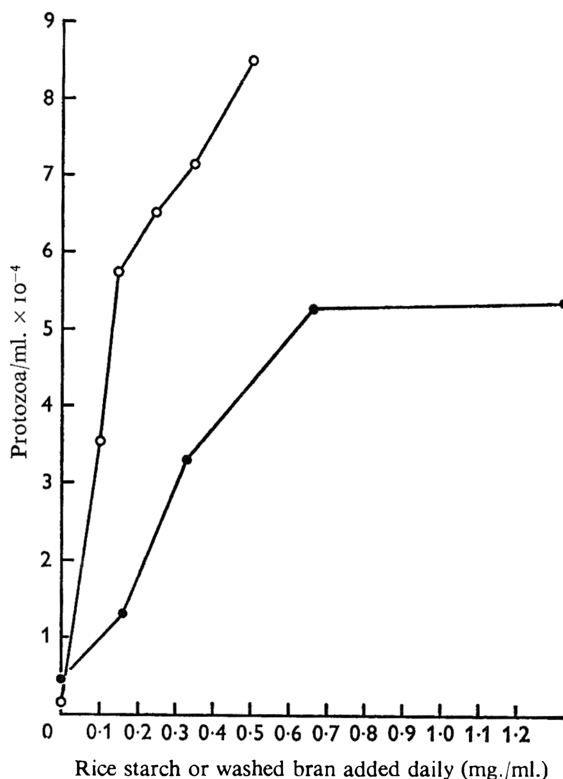

Fig. 1. Effect of quantity of rice starch or washed bran added daily on the steady-state population density of *Entodinium simplex*. The experiment was made under standard conditions on standard medium from which wholemeal flour was omitted. For the experiments on the effect of washed bran (●—●) 0·25 mg. rice starch/ml. was added daily and for those on the effect of rice starch (○—○) 0·6 mg. washed bran/ml. was added each day.

Although bran and rumen fluid both contain B group vitamins it was possible that the bran was acting as a source of thiamine which is known to be heat-labile. The effect of the daily addition of 20 or 200 μg. Difco yeast extract/ml. or 3, 30 or 300 μg.

thiamine/ml. was therefore investigated. These materials had no effect on the numbers of protozoa present after 3 weeks in the presence of rice starch under otherwise standard conditions. However, in one experiment out of four the daily addition of 300 μg. thiamine/ml. increased the number of protozoa by 60 % after 1 week, although after 2 weeks the number had decreased to below that in the absence of thiamine.

Dried grass. In experiments made under standard conditions omission of dried grass from the standard medium, or from media in which the wholemeal flour was replaced by rice starch and washed bran, decreased the number of protozoa by 60 to 70 % after 1 month, though some ciliates survived for more than 4 months.

Autoclaved rumen fluid. In the presence of 0, 3 or 10 % autoclaved rumen fluid (ARF) the numbers of protozoa present in a typical experiment were 8000, 14,800 and 37,000/ml., respectively. Occasionally, however, an increase in the concentration of ARF from 3 to 10 % had no stimulatory effect or was slightly inhibitory. Table 1 shows the effect of various rumen fluid fractions on the population density in an experiment in which double the standard concentration of wholemeal flour was stimulatory. In a series of experiments under standard conditions replacement of ARF by protozoa-containing autoclaved rumen fluid (PARF) or autoclaved bacteria (AB) had a small and variable effect ($\pm$ 30 %) on the number of protozoa; but autoclaved supernatant rumen fluid (AS) always decreased the number to approximately half. This indicated that, as with *Entodinium caudatum* (Coleman, 1960), particulate matter was a better substrate for protozoal growth than soluble material.

In view of the findings of Jarvis & Hungate (1968) that the addition of a protozoal extract (Clarke & Hungate, 1966) to the medium was stimulatory to the growth of *Entodinium simplex*, it was important to investigate the effect of this extract on the growth of this ciliate under the conditions used for the present experiments. The experiment was made in the standard medium in which the wholemeal flour was replaced by rice starch (0·25 mg./ml. daily) and washed bran (0·3 mg./ml. daily). After maintenance for 3 weeks under standard conditions there were 22,000 ciliates/ml. in the presence of 10 % protozoal extract (prepared as described by Clarke & Hungate, 1966) as compared with 51,000/ml. in the presence of 10 % ARF. The addition of both materials together did not increase the number of protozoa.

Basal mineral salts. The standard mineral salt solution which contained phosphate and $NaHCO_3$/100 % CO_2 buffers was replaced by the mineral salt solution of Coleman (1958), which contained only phosphate buffer + acetate and was equilibrated with 95 % (v/v) N_2 + 5 % (v/v) CO_2; this produced very variable results. In some experiments the protozoa died rapidly and in others they grew as well as in the standard medium. This indicated that these ciliates grew as well in the presence of 5 % CO_2 as with 100 % CO_2 provided that they survived the change of medium. *Entodinium simplex* did not survive in the medium for *E. caudatum* (Coleman, 1960), which contained rice starch as source of carbohydrate and also chloramphenicol, unless this was supplemented with 0·6 mg./ml. washed bran daily, under which conditions 50 to 100 % of the numbers of protozoa present in the standard medium were found.

Temperature. Entodinium simplex was very sensitive to change in the growth temperature and after 1 week at 33° or 41° under otherwise standard conditions the numbers had decreased to 100 and 23,400/ml., respectively, as compared with 33,300/ml. at 39°. No protozoa survived after 1 week at 44°.

Growth from small inocula

In the experiments described above the minimum number of protozoa under standard conditions was usually greater than 10,000/ml. after the dilution of a culture, and it was of interest to measure the growth of the protozoa from smaller inocula. Figure 2 shows growth curves for *Entodinium simplex* on standard medium and on standard medium with double the normal concentration of wholemeal flour from a 10% (v/v)

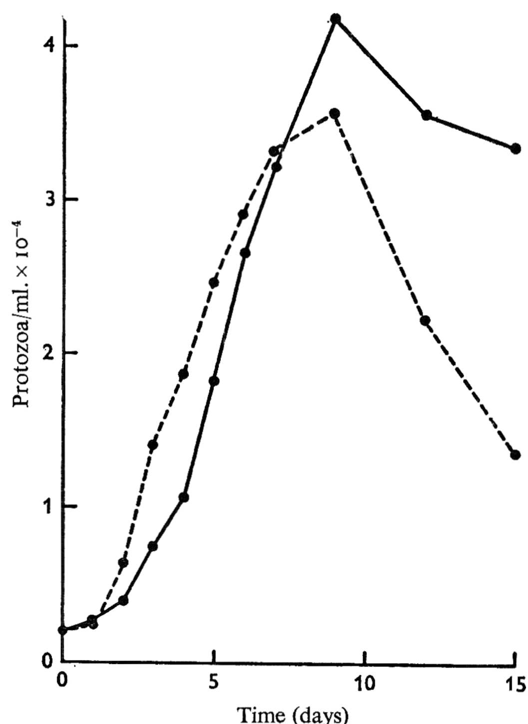

Fig. 2. Growth of *Entodinium simplex* from a small inoculum. The protozoa were maintained under standard conditions except that the cultures were not diluted with fresh medium twice each week. ●—●, Standard medium (i.e. daily addition of 0·5 mg. wholemeal flour/ml.); ●---●, standard medium with double the quantity of wholemeal flour (i.e. 1·0 mg./ml. daily).

inoculum of a stock culture (1800 ciliates/ml.). The generation time over the period of most vigorous growth with the daily addition of 0·5 mg. and 1·0 mg. wholemeal flour/ml. was 2·0 and 1·6 days, respectively. However, once the maximum population density had been reached, the protozoa died more rapidly in the presence of the higher wholemeal flour concentration and this culture became more turbid because of bacterial growth on the excess starch. It was possible to maintain the protozoa on the standard medium with the daily addition of wholemeal flour (0·25 mg./ml. daily) + dried grass, by serial transfer of a 10% (v/v) inoculum once a week; one such culture was continued for over 30 weeks. Unfortunately this was not a reliable method for maintaining stock cultures because occasionally the protozoa died for no obvious reason.

DISCUSSION

Several authors have reported the successful cultivation of rumen entodiniomorphid protozoa in Great Britain, the United States of America and New Zealand but the growth conditions used have varied considerably. None of the workers in the U.S.A. (i.e. Hungate, 1942, 1943; Gutierrez & Davis, 1962; Mah, 1964; Jarvis & Hungate, 1968) have found it necessary to use rumen fluid in their media and, with the exception of Jarvis & Hungate (1968), have also found the addition of reducing agents to be unnecessary. In contrast those working elsewhere (i.e. Coleman, 1960; Clarke, 1963) have been unable to obtain good protozoal growth in the absence of either of these materials. Jarvis & Hungate (1968) also found that soluble materials such as bovine serum and a protozoal extract increased the protozoal growth rate for a few days, whereas in the experiments described above with *Entodinium simplex* and previously with *E. caudatum* (Coleman, 1960) particulate matter (e.g. bran, washed mixed rumen bacteria) was more stimulatory than soluble materials and was effective for at least several months. It seems probable that soluble compounds are stimulatory for a few days before bacteria which grow on the new substrate have been selected and have grown heavily in the protozoal culture. In the present author's experience heavy bacterial growth has always been associated with a decreased number of protozoa/ml., possibly because of a decrease in pH value and the production of compounds toxic to the protozoa. In contrast, the use of solid food materials which were comparatively resistant to bacterial attack and were engulfed by the protozoa resulted in high stable population densities of entodinia. The cause of the other differences reported by authors in different countries is less apparent. The workers in U.S.A. and New Zealand used cattle ciliates, whereas the present author has always used those from sheep; perhaps apparently identical species of ciliate from different ruminants may behave differently in culture. It is also possible that initially identical ciliates may have evolved differently in host ruminants living in widely separated countries under different climatic conditions and therefore behave differently in culture.

Although the initial isolation of *Entodinium simplex* was made by using wholemeal flour as carbohydrate source, a mixture of rice starch + washed bran was subsequently found to give much better growth, especially after a few weeks of adaptation to this medium. Since many of the wheat starch grains were too large to be engulfed by *E. simplex* and must have been fermented by the bacteria in the cultures, the successful use of rice starch was probably associated with its complete utilization by the protozoa. The role of the bran in these cultures is uncertain, but it must supply some material, possibly a growth factor, not present in the starch or the bacteria living in the cultures. Unfortunately it was not possible to show with certainty a stimulation of protozoal growth by the addition of a solution of a mixture of growth factors (i.e. yeast extract) or of thiamine.

I wish to thank Mr G. A. Embleton, who inserted permanent rumen canulae in the sheep used as a source of rumen fluid, the members of the Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, for helpful criticism and advice, and Miss B. C. Hanzl for valuable technical assistance.

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Septal Pores in *Endomycopsis platypodis* and *Endomycopsis monospora*

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SUMMARY

In ultra thin sections of hyphae of the yeast species *Endomycopsis platypodis* and *E. monospora* septal pores were observed with the electron microscope. The pores are enclosed by a swelling of the cross-wall. Electron-dense bodies plug the pores; these bodies are surrounded by a membrane which is connected with the endoplasmic reticulum and which also connects the plugs at both sides of the cross-wall via the pore.

INTRODUCTION

The ascogenous yeast species *Endomycopsis platypodis* Baker et Kreger-van Rij (1964) and *E. monospora* Saito (1932) produce abundant true mycelium as well as budding cells, a combination of features which characterizes the genus *Endomycopsis*. Classification of these species in *Endomycopsis* is, however, not generally accepted. Fiol (1967) transferred *E. platypodis* to the genus *Hansenula* as defined by Wickerham (1951). This genus comprises all nitrate-positive species with hat- or saturn-shaped spores; and Wickerham included in it both yeasts which do and yeasts which do not form true mycelium. Boidin, Pignal & Besson (1965) followed him by classifying species with and without true mycelium in the similar genus, *Pichia*, members of which do not utilize nitrate. One of these species was *Pichia* (*Endomycopsis*) *fasciculata*. These authors did not consider that the formation of true mycelium was a suitable character for distinguishing between genera. Although this point of view either necessitated the abolition of the genus *Endomycopsis* or required its redefinition for those species which were not reclassified, neither measure was undertaken. Unlike Wickerham or Boidin and his colleagues, Kreger-van Rij (1964) retained the genus *Endomycopsis* in its original shape, because most of its species have different features from those of species of the genera *Hansenula* or *Pichia* in addition to the formation of abundant true mycelium. Adequate studies have yet to be made on changes in fine structure associated with the formation of true mycelium on the one hand and with pseudomycelium on the other. The only ultrastructural studies of mycelium in Endomycopsis species are probably those of Takada, Yagi & Hiroaka (1965), who described the occurrence of plasmodesmata in the cross-walls of *E. fibuligera*, and of Besson (1967),

who observed plasmodesmata in *E. capsularis*. In the present work the observation of septal pores in *E. platypodis* and *E. monospora* is reported. Some additional species, thought to be related to *E. platypodis* and *E. monospora*, have been examined for comparison.

METHODS

The following strains were used:
Endomycopsis platypodis Baker et Kreger-van Rij, CBS 4111; *E. platypodis* Baker et Kreger-van Rij, Y-6101 from Dr L. J. Wickerham, CBS 5560; *E. monospora* Saito, CBS 2554; *E. monospora* Saito, strain *Endomycopsis fasciculata* Batra, CBS 5514; *E. bispora* (Beck) Dekker, CBS 1890; *E. fibuligera* (Lindner) Dekker, CBS 5190; *E. vini* Kreger-van Rij, CBS 4377; *Cephaloascus fragrans* Hanawa, strain *Ascocybe grovesii* Wells.

The organisms were grown on malt agar, corn meal agar or on a medium containing 0·3 % yeast extract, 0·5 % peptone, 1 % glucose and 2 % agar. Young cultures were fixed (1) according to the method of Ryter & Kellenberger (1958) in 1 % osmic acid in veronal + acetate buffer (pH 6) for 16 hr, and, after washing with Kellenberger buffer, in 0·5 % uranyl acetate for one hr, both at room temperature, or (2) in 1·5 % aqueous unbuffered $KMnO_4$ solution for 20 min. at room temperature. After dehydration in an acetone series, embedding was done in Vestopal W at 60°. Ultra thin sections were cut with a diamond knife on an LKB Ultrotome. Part of the sections was stained with lead citrate (Reynolds, 1963) for 3 min. or with 1·5 % $KMnO_4$ solution for 30 min. They were examined with a Philips EM 100 electron microscope.

RESULTS

The hyphae of *Endomycopsis platypodis* in serial sections show septa with central pores (Fig. 1; Pl. 1 and 2). The septa are slightly bulging. The edge of the septum around the pore is swollen. The swelling diminishes abruptly at the concave side and gradually at the convex side of the cross-wall. Together with the pore the swelling constitutes the greater part of the septum. The central layer of the septal plate is electron-light, the adjacent layers including the swelling are relatively electron-dense. In longitudinal sections the swelling shows a fibrillar structure (Pl. 2, fig. 8); in cross-sections through the cross-wall the fibrils are observed as concentric rings (Pl. 2, fig. 9). The plasmalemma, showing as two thin dark lines with a light interspace, lines the protoplast and is continuous through the pore. Two dark bodies plug the pore at each side. They are surrounded by a membrane which envelops them both; the connexion of the plugs is formed by a narrow channel through the pore (Pl. 1, fig. 4). The double membrane of the endoplasmic reticulum lies parallel to the lateral cell wall and the cross-wall. Over the pore the endoplasmic reticulum is continuous in such a way that the plugs seem to be dilatations of it (Pl. 1, fig. 2 and 4). Connexions between the membrane of the plugs with other endoplasmic reticulum than the cortical form were also found to occur (Pl. 2, fig 7 and 11). Occasionally, a continuous double membrane of endoplasmic reticulum, connected with the cortical endoplasmic reticulum, was observed lying over the plugged pore (Pl. 1, fig. 5), but this was never in the shape of the thicker, perforated endoplasmic reticulum which forms the septal pore cap of the Basidiomycetes. The plug at the concave side of the cross-wall was generally observed to be lying against the wall, the one at the convex side at some distance from the wall.

The septal pores were observed in the hyphae of the yeast. These hyphae do not split up into arthrospores. Between the asci, which are situated terminally or laterally on the hyphae, often in short chains, pores were also present. In budding cells, whether loose or in small chains, the wall formed between mother cell and bud was usually thin. Budding cells formed on the hyphae which did not turn into asci (blastospores) have not yet been examined.

The swellings in the septa were clearly seen by light microscopy (Pl. 3, fig. 15). Formation of a new septum, observed from a first vague indication to a distinct line, occurred within 8 min. It took about an hour until a swelling became visible.

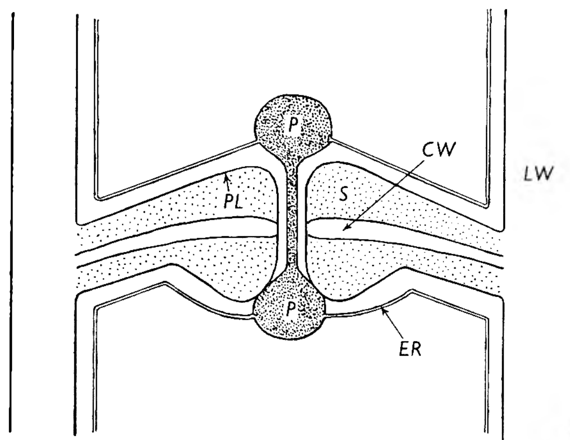

Fig. 1. Diagram of a longitudinal section of a septal pore in *Endomycopsis platypodis* and *E. monospora*. *LW* = lateral wall, *CW* = cross-wall, *S* = swelling, *PL* = plasmalemma, *P* = plug, *ER* = endoplasmic reticulum.

Septal pores were observed in the two strains of *Endomycopsis platypodis* studied, in the strain of *E. monospora* (CBS 2554), and in a strain of *E. fasciculata*, which is considered to be synonymous with *E. monospora*. In *E. bispora* no septal pores were observed. The hyphae of *Cephaloascus fragrans* showed simple pores of the Ascomycete type (Pl. 3, fig. 12). No Woronin bodies but small dense granules as described by Brenner and Carroll (1968) were found in the material examined (Pl. 3, fig. 13). The strains of *E. fibuligera* and *E. vini* (Pl. 3, fig. 14) had plasmodesmata as connexions between the cells of the hyphae.

DISCUSSION

A central pore surrounded by the swollen edge of the cross-wall observed in hyphae of *Endomycopsis platypodis* and *E. monospora* greatly resembles the pores found in Basidiomycetes (Girbardt, 1958, 1961; Moore & McAlear, 1962; Bracker & Butler, 1963, 1964; Wilsenach & Kessel, 1965; Giesy & Day, 1965; Berliner & Duff, 1965; Hyde & Walkinson, 1966), the 'dolipore' of Moore & McAlear (1962). In contrast with the Basidiomycete-type pore, no pore cap was observed in the yeasts. On the other hand, all pores were found to be plugged. For the Basidiomycetes, Bracker & Butler (1963) are the only authors to mention the occurrence of plugs, which they found in hyphae from old cultures of *Rhizoctonia solani*. The plugs were amorphous and electron-dense while the inside of the pore was also occluded with electron-dense

material. The picture of these plugs does not show a membrane surrounding them. In Ascomycetes, Woronin bodies have been described as plugs of septal pores in *Fusarium spp.* (Reichle & Alexander, 1965) and in *Ascodesmis sphaerospora* (Brenner & Carroll, 1968). These bodies are electron-dense and they are surrounded by a single membrane. Brenner & Carroll (1968) suggested that they may be dilated cisternae of the endoplasmic reticulum. The plugs of the yeasts bear some resemblance to the Woronin bodies. However, the number of the latter at each side of the pore may be two to four in electron micrographs (Reichle & Alexander, 1965) while in the yeasts only one plug was found at each side.

According to Bracker & Butler (1963), the septal swelling differs chemically and physically from the walls. In adjacent cells without protoplasm the cross-wall was found to be intact, but the swelling not present. We observed the same in dead hyphae of *Endomycopsis monospora*.

Two kinds of septal pore have been found in the fungi, namely the Ascomycete type and the Basidiomycete type. Several authors are inclined to attach taxonomic importance to this feature, and to consider its phylogenetic significance. In this respect, it is surprising to find in yeast species, classified as Ascomycetes, a type of pore which resembles that of the Basidiomycetes. On the other hand, lack of a pore cap on this type of pore is a new observation, as is the probably permanent presence of plugs.

The yeast strains in which the dolipores have been observed belong to two different species which are, however, similar in several respects. They differ with respect to the ability to utilize nitrate, so that authors who do not accept the genus *Endomycopsis* must classify the two species in different genera, as *Hansenula platypodis* and *Pichia monospora*. It remains to be seen whether dolipores occur in the hyphae of other Hansenula and Pichia species; and if this were not so, it must be asked to which property, presence of dolipores or utilization of nitrate, priority should be given for reclassification. For the present, it is preferred to retain the species in question in the genus *Endomycopsis*.

Dr L. J. Wickerham (personal communication) drew attention to the resemblance between the aerial ascophores of *Cephaloascus fragrans* and the hyphae that bear asci in *Endomycopsis platypodis*. He believed that there is a somewhat distant relationship between the two species, although he was quite aware of the differences in structure of the ascophores of *C. fragrans* and *E. platypodis*. The finding of simple pores of the Ascomycete type in *C. fragrans* by Besson (1967) and by the present authors and of dolipores in *E. platypodis* emphasizes the differences between the two species.

In a third Endomycopsis species, *E. bispora*, which Kreger-van Rij (1964) considered to be related to *E. platypodis* and *E. monospora*, no dolipores were found. Two other related species, *Endomycopsis fibuligera* and *E. vini*, did not show dolipores either. We found plasmodesmata in the cross-walls of *E. fibuligera* as Takada *et al.* (1965) did, and *E. vini*, which closely resembles *E. fibuligera*, also had plasmodesmata.

There is no doubt that the genus *Endomycopsis* in its present definition (Kreger-van Rij, 1964) is heterogeneous. There is, however, something to be said for retaining this taxon until several of its features, such as the production of true mycelium and buds, have been more closely examined.

We are indebted to Dr J. Boddingius for making the light micrograph. Our thanks are due to Dr J. A. Barnett for corrections of the English text. The first author wishes to acknowledge the hospitality of the Laboratory of Ultrastructural Biology of the State University of Groningen.

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EXPLANATION OF PLATES

Symbols: *CW* = cross-wall, *S* = swelling, *PL* = plasmalemma, *P* = plug, *ER* = endoplasmic reticulum. In Fig. 1 to 14 the marker represents 0·5 μ, in Fig. 15, 5 μ. The figures of Plate 1 and 2 are hyphae fixed with $KMnO_4$ and post-stained with lead, unless otherwise indicated.

Plate 1

Fig. 1 to 5. Longitudinal sections of hyphae of *E. platypodis*.

Fig. 1. Six serial sections through a septal pore.

Fig. 2. Section showing connexions between plug- and ER membranes. Note continuity of plasmalemma through the pore.

Fig. 3. Section of material fixed with osmic acid and uranyl acetate, and post-stained with lead.

Fig. 4. Section through a pore showing continuity of ER with plug membrane and connexion of the two plugs via the pore.

Fig. 5. Cortical ER lying over the plug.

Plate 2

Fig. 6 to 7 and 10 to 11 are longitudinal sections of hyphae of *E. platypodis*, Fig. 8 of *E. monospora*.

Fig. 6. Section showing the position of the plugs, the one at the concave side close to the wall, the one at the convex side at some distance from it.

Fig. 7. The ER system is more complicated and at several points connected with the plugs.

Fig. 8. The swelling shows a fibrillar structure.

Fig 9. In cross-section, the swelling (S) shows more or less a structure of concentric rings. Within the pore the tubular connexion of the two plugs is visible. The swelling is partly surrounded by the plasmalemma and by strands of endoplasmic reticulum.

Fig. 10. Oblique section through a pore with only one plug visible. The ER lies parallel to the cross-wall.

Fig. 11. Similar section through the same pore with both plugs visible.

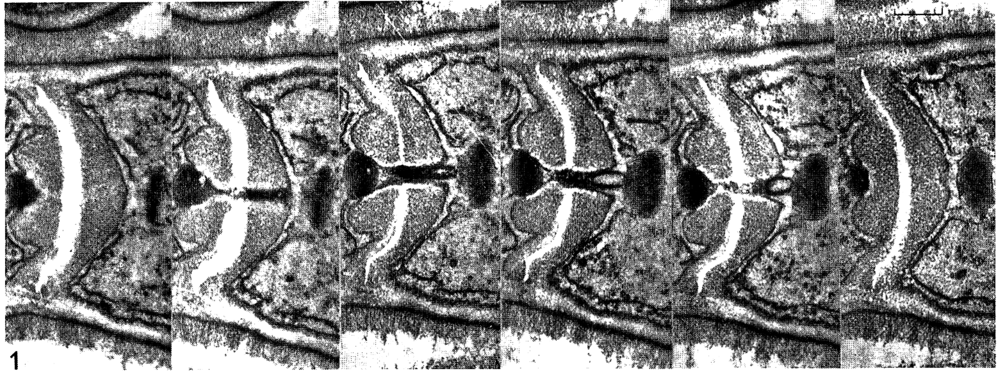
Plate 3

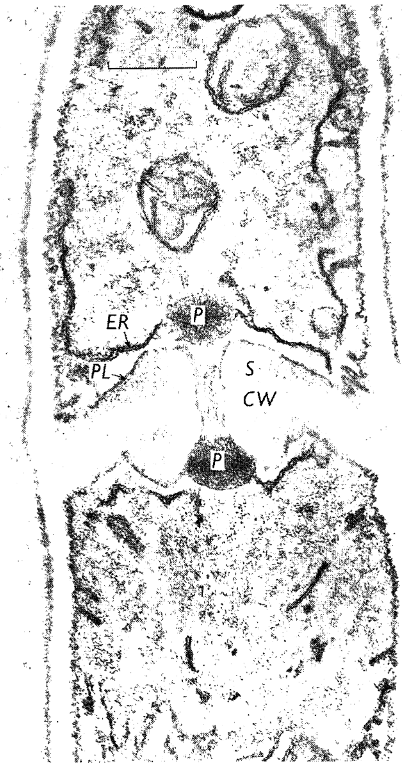
Fig. 12 to 14 are fixed with osmic acid and uranyl acetate and post-stained with lead.

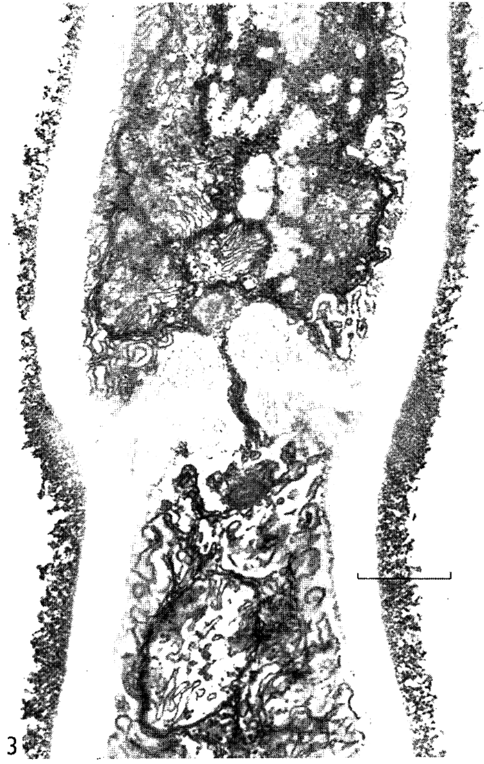
Fig. 12 and 13. Longitudinal sections through hyphae of *Cephaloascus fragrans*. A simple central pore is present, with small dense granules in the vicinity, clearly seen in Fig. 13.

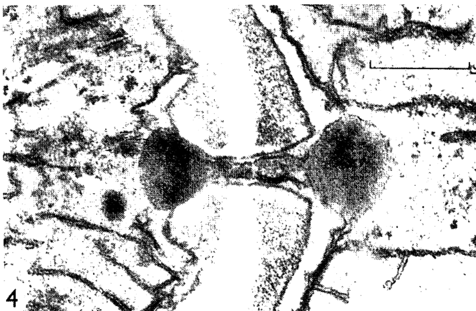
Fig. 14. Longitudinal section through a hypha of *Endomycopsis vini*. Several plasmodesmata are visible connecting the protoplasts of adjacent cells.

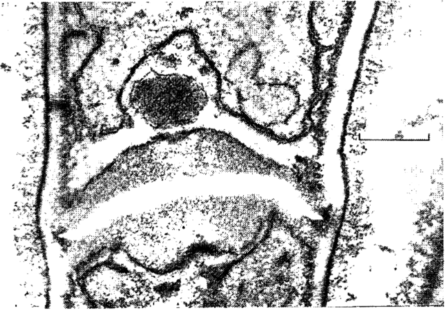
Fig. 15. Light micrograph of a hypha of *Endomycopsis platypodis*. In the cross-wall the swelling shows as a dark ring with a light centre.











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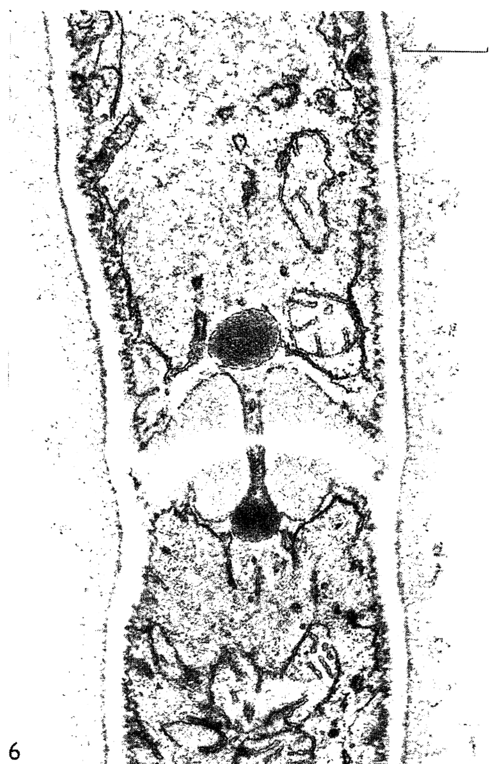

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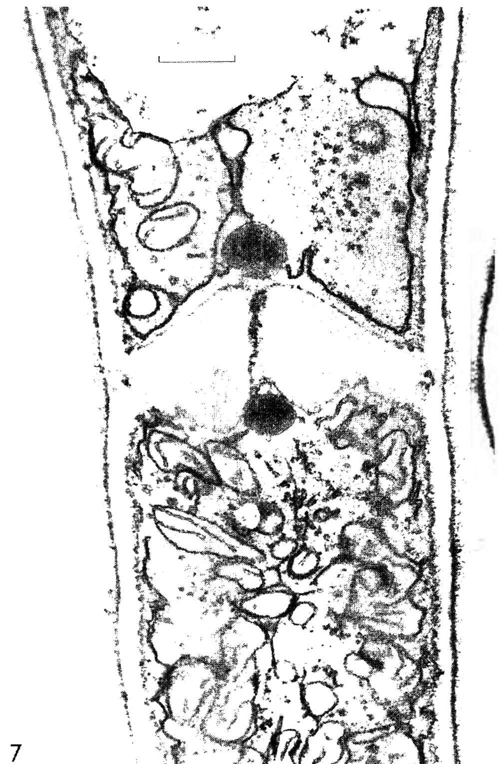

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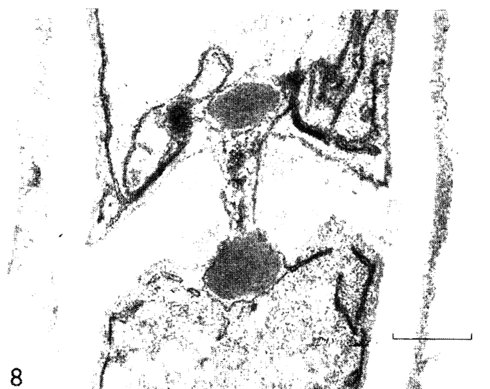

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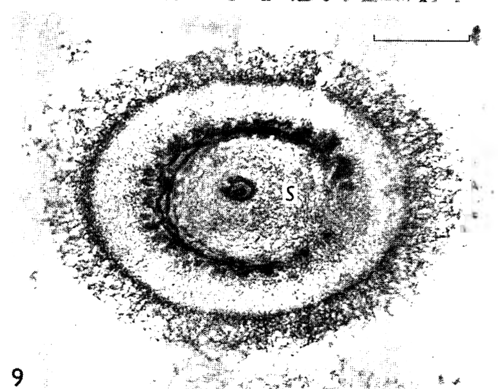

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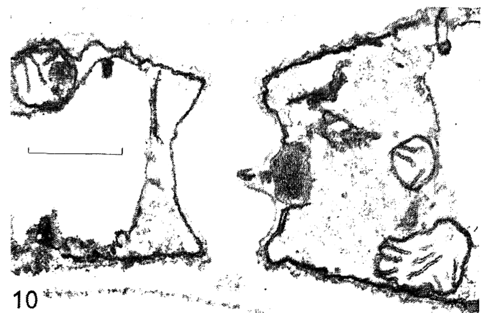

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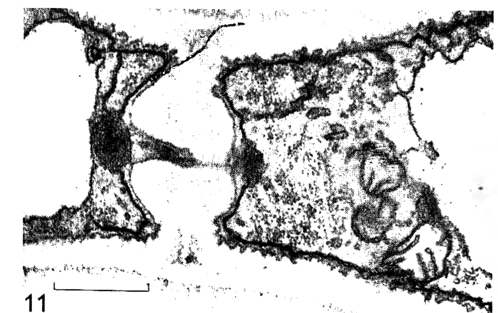

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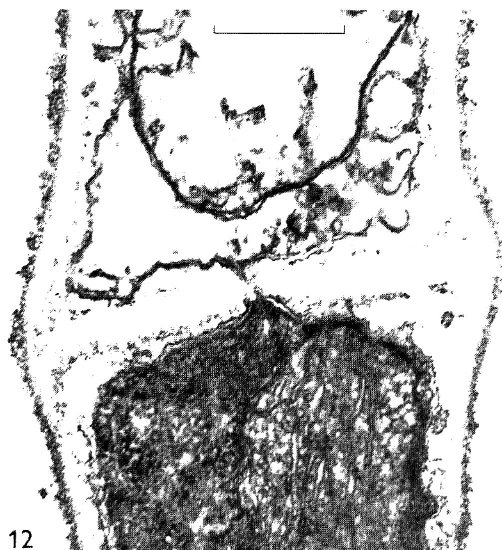
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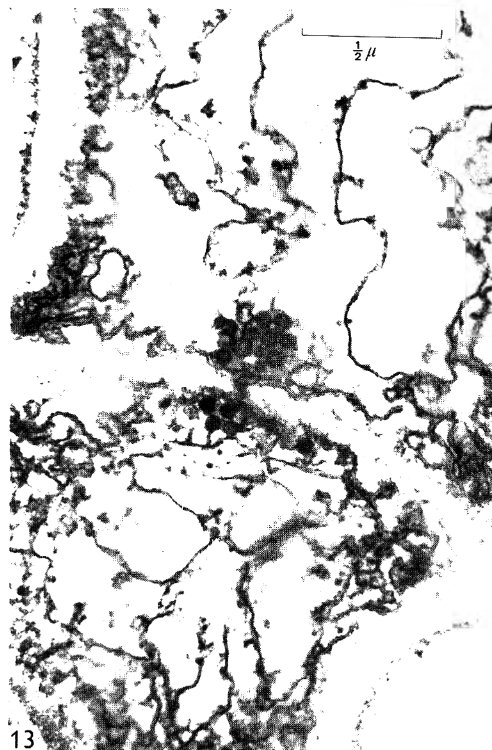

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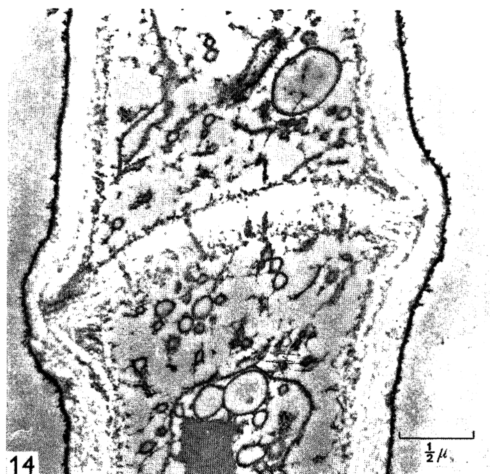

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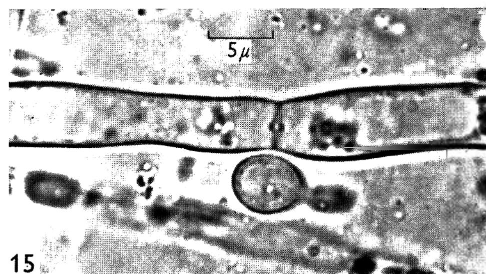

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N. J. W. KREGER-VAN RIJ AND M. VEENHUIS

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Split-dose Irradiation of *Escherichia coli* in the Absence and Presence of Mercaptoethylamine

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SUMMARY

A single dose of γ-radiation to *Escherichia coli* B gave a semi-logarithmic dose-response curve. A second irradiation 3, 6, or 18 hr after a priming dose of 8 krad showed that the cells had become more radio-sensitive during the incubation between irradiations.

When mercaptoethylamine was present during irradiation, protection against the lethal effects of the γ-radiation occurred. After a priming dose to the bacteria in the presence of the radio-protective chemical, and incubations for 3, 6, or 18 hr in its absence, a second irradiation in the absence of the chemical showed them to be much more radio-resistant than the normal unprotected *Escherichia coli* B. This indicates that the presence of the protector during irradiation alters the type of damage induced or the development and repair of that damage.

INTRODUCTION

In many types of cell significant differences can be demonstrated between the survival curves obtained following single and split-dose irradiations with γ rays. These differences may reflect repair of radiation damage during the interval between split-dose irradiations, synchronization of cells in a certain stage of the cell cycle at the time of the second irradiation, or changes in metabolism which affect the response of the cells to a second irradiation (Elkind & Sutton, 1959; Elkind, 1966).

Radioprotective chemicals are agents which by their presence during irradiation decrease the effects of sparsely ionizing radiations on living cells (Bacq, 1965). Post-irradiation repair of radiation damage induced in the presence of protector has received little attention, despite the possibility that the protective chemical may modify the initial radiation injury in such a way that the post-irradiation restoration may also be modified.

The response of *Escherichia coli* B to single and split-dose irradiations in the absence and presence of the chemical protector mercaptoethylamine (MEA) was therefore investigated, in order to determine the effects of the protector on the development of damage following irradiation.

METHODS

An inoculum of *Escherichia coli* B from a Difco nutrient agar slope was transferred to 500 ml. of Oxoid CM. 1 nutrient broth. After shaking vigorously, this was incubated at 37° for 24 hr to obtain a culture in stationary phase (Elias, 1961). The bacterial concentration was then about 2×10^8 bacteria/ml. A 45 ml. volume of the culture was centrifuged and the bacteria added to 45 ml. of an aerated Oxoid CM. 1 nutrient broth + 0·85 % saline mixture (with a broth: saline ratio of 1:100) at 0°.

For experiments not involving MEA this suspension was diluted 1/100 in the saline broth mixture and left for 1 hr at 0°. When the bacteria were to be protected, the chemical MEA was added to the suspension to give a final concentration of 0·04 M (Ginsberg, 1966) and left for 1 hr at 0° before irradiation. The dilution of these protected bacteria by 1/100 occurred after irradiation to minimize any effects of the protector in the later part of the experiment.

Aeration of the bacterial culture and suspensions was done by continuous shaking during the incubations before irradiation and between irradiations.

The suspensions were irradiated at 0° in a conical flask with a cotton-wool stopper, using a 9000 C ^{60}Co-γ source, giving a dose of 815 rad/min. as estimated by Fricke ferrous sulphate dosimetry. Serial samples were taken from the suspension after known irradiation doses, and the culture was aerated by shaking each time a sample was taken (at approximately 5 or 10 min. intervals).

The samples from the irradiated bacterial suspensions were diluted, plated out in triplicate on Difco nutrient agar plates and incubated for 24 hr at 37°. Those colonies which were visible to the naked eye were counted, and the criterion of survival of an irradiated bacterium was taken as the ability to form a visible colony.

Recovery of the cell suspensions was investigated following initial radiation doses of 8 krad in the absence of MEA and 12 krad (in the presence of MEA): these doses gave about 20 to 30 % survival. In one experiment a dose of 12 krad was also given to a culture lacking MEA. The non-protected samples were incubated in the saline broth mixture for 3, 12, or 18 hr between the two irradiations, at a temperature of 18° which gives a reasonable opportunity for restoration (Stapleton, 1955; Hollaender & Stapleton, 1953). Samples which had been irradiated in the presence of MEA were immediately centrifuged at 0 to 1° and resuspended in the saline + broth mixture; this was done either one or three times. The suspension was then incubated at 18° for 3, 12, or 18 hr before the second irradiation in the absence of MEA.

Each experiment was repeated three times, except for the 12 hr incubation after irradiation in the presence of MEA (repeated twice) and the experiments involving the preincubation of MEA (once), and three repeated centrifugations before incubation (once).

RESULTS

Single-dose experiments

The effects of a single dose γ-irradiation on bacterial survival were examined. When MEA was not present (Fig. 1 *a*) the dose-response took the form of a single exponential function, with the D_0 (i.e. the dose required to reduce the number of surviving bacteria by 63 %) being $6·5 \pm 0·6$ krad.

When the bacterial suspension was incubated with MEA at 0° for 1 hr, centrifuged, resuspended in a saline broth mixture without MEA and immediately irradiated at 0°

the form of the dose response curve (Fig. 1 *c*) did not differ significantly from that of the bacteria which had not been pre-incubated with MEA (Fig. 1 *a*). The D_0 was in this event 6 ± 1 krads. This indicates that after the single centrifugation and resuspension the concentration of MEA associated with the bacteria was not sufficient to alter their dose response significantly.

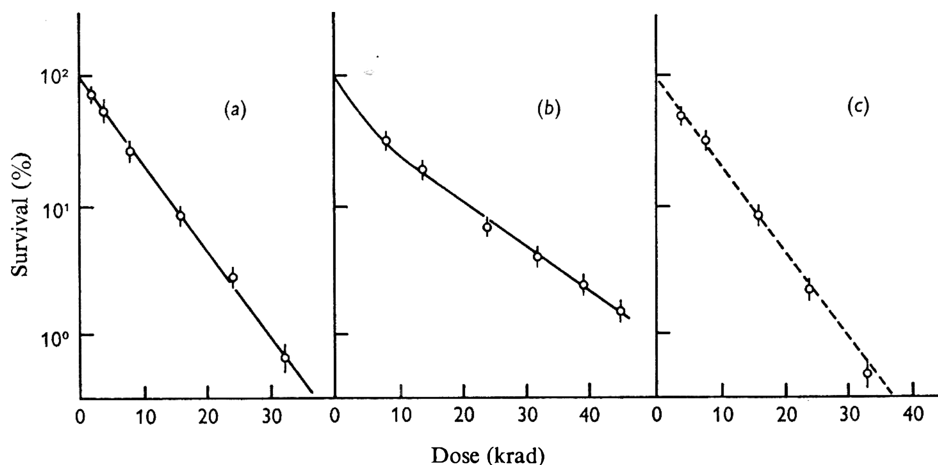

Fig. 1. Single-dose survival curves after the ^{60}Co-γ irradiation of *Escherichia coli* B in the absence and presence of mercaptoethylamine. (*a*) No mercaptoethylamine present during irradiation; (*b*) Irradiation in the presence of 0·04 M-mercaptoethylamine; (*c*) irradiation after one washing of the bacteria following 1 hr preincubation with 0·04 M-mercaptoethylamine. The curve of (*a*)—no mercaptoethylamine present during irradiation—is superimposed in this graph, thus – – –.

In the presence of MEA an initial rapid drop in survival predominated until about 12 krad and at higher doses the decrease in survival with dose became slower, with D_0 equal to 13 ± 2 krads. Extrapolation of this slower exponential decrease to the ordinate gave an intercept at about 35 to 45 % (Fig. 1 *b*). This suggests that two different populations coexist in the presence of MEA: first, a population comprising 55 to 65 % of the total with a D_0 not greatly different from that of unprotected bacteria, and secondly, a population comprising 35 to 45 % of the total with D_0 double that of unprotected bacteria. The dose-reduction factor for this second population is thus about 2 (i.e. D_0 in presence of MEA/D_0 in absence of MEA).

Split-dose experiments: no MEA.

The effects of splitting the irradiation dose by 3, 12, or 18 hr were examined (Fig. 2). The curves for the second irradiation all have a 'shoulder', i.e. the slope of the curve increases with dose (the single dose curve is shown for comparison). The slope of the dose-response curve at low doses was not significantly different from that of the single dose curve, except after an 18 hr interval when the initial D_0 was about 9 krad. At second doses higher than about 20 krad the slope of the curve was much steeper, with the D_0 lying between 3·0 and 4·0 krad in each instance; this demonstrates a sensitization of the bacteria to the radiation at high doses, relative to their sensitivity as demonstrated by the single-dose experiments.

The effect of a second dose of radiation 12 hr after an initial dose of 12 krad (i.e. the same does as given to protected bacteria) was similar to that after an initial dose of 8 krad.

Split-dose experiments: MEA present during first irradiation

The dose resonse curves for a second irradiation in the absence of MEA (following incubations at 18° for 3, 12, or 18 hr after the first irradiation in the presence of MEA) again demonstrated a shoulder after low doses (Fig. 3). At higher doses (greater than 20 to 25 krads) the curve became exponential, approximately parallel to the single-dose curve in the absence of MEA, and the D_0 values for the three curves at a high dose all lay between 5·5 and 7 krad. Thus no sensitization occurred in this case, above the radio-sensitivity of the bacteria to a single dose.

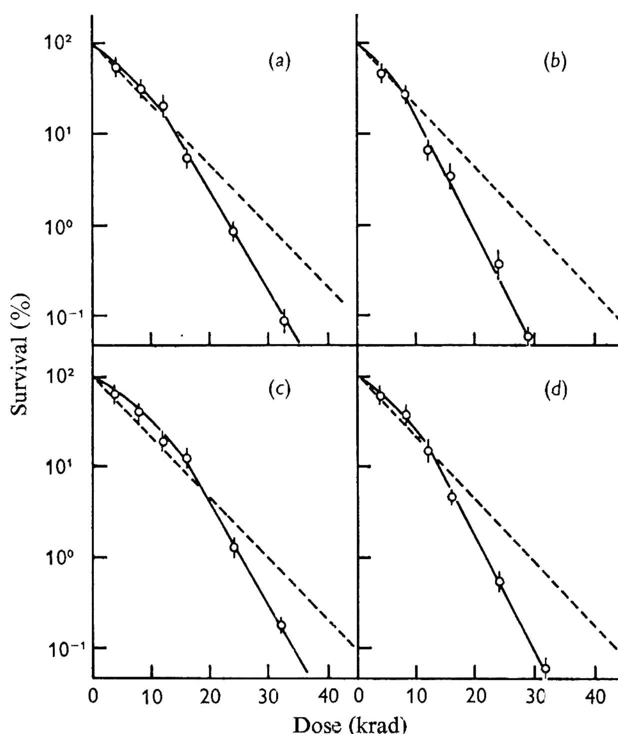

Fig. 2. The effect of a second ^{60}Co-γ irradiation on the survival of *Escherichia coli* B. No mercaptoethylamine was present during irradiation. The single-dose response curve of (*a*) —no mercaptoethylamine present during irradiation—is superimposed on the graphs for comparison thus – – –. (*a*) 3 hr interval between irradiations, following a primary dose of 8 krad; (*b*) 12 hr interval between irradiations, following a primary dose of 8 krad; (*c*) 18 hr interval between irradiations, following a primary dose of 8 krad; (*d*) 12 hr interval between irradiations following a primary dose of 12 krad.

Bacteria which had MEA present during the first irradiation were therefore less sensitive to a second dose than the bacteria which were not incubated with MEA during the first irradiation. To investigate the possibility that some MEA may remain in the bacteria after one centrifugation and resuspension, thereby causing protection during the

second irradiation, the bacteria in one experiment were centrifuged and resuspended three times at the beginning of a 12 hr interval at 18°: the results of a second irradiation of these bacteria (Fig. 3*d*), showed once again a shoulder, and a final D_0 of 6 krad.

Cell division during split-dose experiments

The suspending medium during the interval between irradiations was always the saline-broth mixture. No bacterial division occurred in any suspension which had MEA present during the first irradiation, nor in the suspension without MEA which was left for 3 hr between irradiations, as estimated by plating-out techniques.

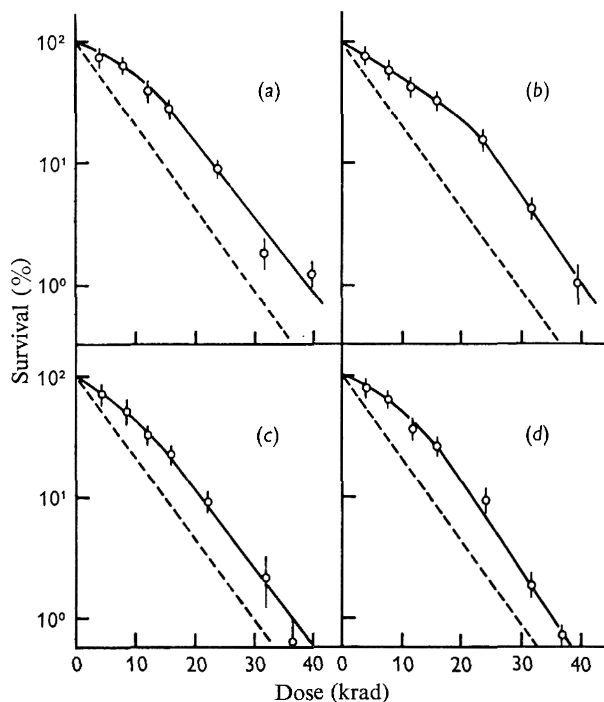

Fig. 3. The effect of a second ^{60}Co-γ irradiation in the absence of MEA on the survival of *Escherichia coli* B after a priming dose of 12 krad in the presence of 0·04 M-mercaptoethylamine. The single-dose response curve of Fig. 1 (*a*)—no mercaptoethylamine present during irradiation—is superimposed on the graphs for comparison, thus - - -. (*a*) 3 hr interval between irradiations; bacteria washed *once* after first irradiation; (*b*) 12 hr interval between irradiations; bacteria washed *once* after first irradiation; (c) 18 hr interval between irradiations; bacteria washed *once* after first irradiation; (*d*) 12 hr interval between irradiations; bacteria washed *three times* after first irradiation.

The bacteria initially irradiated in the absence of MEA had increased in number by factors of 10 and 32 at the end of 12 and 18 hr incubations respectively, representing concentrations 4 and 12 times as great as that of the bacterial suspension before irradiation. Thus the increase cannot be caused merely by repair of damage during incubation (Stapleton, 1955) but must involve cell division.

Toxicity of MEA

Because MEA is a toxic chemical, a test was made to determine whether it was damaging the bacteria, thereby affecting their measured survival. They were incubated with MEA at 0 to 1° for 1 hr, and were then diluted and plated out.

The concentration of 0·04 M-MEA (used in these experiments) did not measurably affect survival; no significant decrease in survival occurred at concentrations up to 0·25 M.

DISCUSSION

The dose-response curve for the single-dose irradiation in the presence of MEA demonstrated that there were probably at least two different populations of bacteria, one showing little protection and one showing considerably greater protection. This is an unusual finding, as other workers appear to find a fairly uniform protection of all the cells (Elias, 1961; Ginsberg, 1966; Comroy & Adler, 1962), and it may be caused by differences in the organisms' abilities to take up MEA according to their cell cycle stage at the beginning of incubation with MEA, before division stops (Ginsberg, 1966). The use of high concentrations, relative to the amount of protective chemical, may contribute to this effect. There were about 2×10^8 bacteria per ml. in this study, whereas other workers have used between 4×10^4 and 10^8 bacteria per ml. (Elias, 1961; Hernadi, Valyi-Nagy, Nagy & Seney, 1962; Kohn & Gunter, 1959; Ginsberg, 1966), and therefore they may have had excess MEA present, thereby allowing all the bacteria to take up sufficient MEA to induce radio-protection.

Dose-reduction factors depend on many variables (Elias, 1961; Ginsberg, 1966, Comroy & Adler, 1962): the dose-reduction factor of 2 found for protected bacteria in this study cannot therefore be compared directly with those found by other workers but it may be related to the magnitude of the oxygen effect in these bacteria under these experimental conditions (Comroy & Adler, 1962).

The possibility of variations in oxygen concentrations during the experimental procedures must be considered, as Alper & Moore (1967), using slightly different experimental conditions, found an oxygen enhancement ratio of 2·84 for *Escherichia coli* B. Any consumption of oxygen during the autoxidation of cysteamine should be maximum during the incubation prior to irradiation, when aeration occurs. Decrease of oxygen concentration due to the γ-irradiation should be small, as the dose-rate is low enough to allow diffusion of oxygen into the bactera (Dewey & Boag, 1959). The oxygen consumption of the bacteria, although finite at 0° during the 10 min. irradiation periods, should not be sufficient to induce significant anoxia. The possibility that the oxygen concentrations may alter slightly during irradiation cannot therefore be excluded, but significant decrease in the dose-response curves would require a comparatively high degree of anoxia, with an oxygen partial pressure of the order of 1 % or less (Howard-Flanders & Alper, 1957; Dewey & Boag, 1959).

Experiments were standardized to give equivalent amounts of radiation damage (as measured by cell death) during the first irradiation, and after that the conditions were the same for both previously protected and unprotected organisms. Thus differences in cellular metabolism and radiation responses following a primary irradiation should therefore reflect differences in the type and development of damage induced by the initial irradiation rather than environmental factors after irradiation.

In the split-dose experiments in the absence of MEA the low D_0 obtained after high second doses suggests that the incubation between irradiations renders bacteria less capable of withstanding high radiation doses, while having little effect on their response to low doses. This may be in part due to the bacteria entering a growth phase following irradiation: the bacteria had not actually divided during the 3 hr incubation, but at 12 hr had divided on average 3·2 times. If this explanation is correct, it must be postulated that at 3 hr after 8 krad the bacteria are moving through the DNA synthetic and the G_2 period before division and are therefore rendered more sensitive to the radiation, although Stapleton (1955) found that the radio-sensitivity of *Escherichia coli* B/R only increased when the bacteria entered log. growth phase, and actually decreased at the end of the lag phase. Although it may be possible to investigate the importance of growth phase by allowing these irradiated bacteria to divide until they reach stationary phase (this requiring on average 9 to 10 divisions) and then determining the radiation response, there would have been considerable selection for bacteria showing little radiation damage and a consequent depression in the percentage of heavily damaged bacteria during the divisions; the final culture in stationary phase could not therefore be truly compared with the culture in stationary phase during the first irradiation. Also, considerable intracellular repair of damage may occur, thereby altering the radiation response.

The change in sensitivity after irradiation could also be caused by the development of radiation-induced changes in bacterial metabolism (not connected with division) which could change the organism's response to further radiation lesions.

The response of bacteria to a second irradiation, after irradiation in the presence of MEA, was significantly different from when MEA was not present during the first irradiation, and indicates that the chemical may not have been removed from the bacteria by the washing procedure. However, pre-incubation of the bacteria with MEA, followed by centrifugation and resuspension, did not induce radioprotection, and this suggests that the chemical is readily removed. A similar conclusion may be derived from the fact that repeating the normal washing procedure three times after the first irradiation did not significantly alter the dose-response curve for the second irradiation. Ginsberg (1966) has also shown, using ^{35}S-MEA, that the process of adsorption or absorption is readily reversible and that the chemical is rapidly removed from *Escherichia coli* by washing, when MEA-induced inhibition of DNA synthesis and division ceases immediately and radio-protection also ceases. It seems, therefore, that the additional protection observed in this study is unlikely to be caused by the presence of MEA.

Similarly, the lack of division after irradiation of the bacteria in the presence of MEA does not seem to be caused by a residue of the chemical remaining in the bacteria and it seems therefore that the type of radiation damage occurring in the presence of MEA differs from that occurring in its absence. This difference expresses itself to some extent in the inhibition of division, which may partially explain the protective action of the chemical, in accordance with the view that bacteria have a better chance of recovering from radiation damage if there is a delay in division following irradiation (Sinclair, 1966).

The development of the shoulder on the split-dose curves, with extrapolation numbers in the range 5 to 10, suggests that bacteria after being irradiated in the presence of MEA are altered in such a way that they can withstand a larger amount of radiation

damage before being killed. This is unlikely to be a result of cessation of division, as the bacterial culture for the single experiment in the absence of MEA was also in stationary phase, this latter observation is also confirmed by the experiment involving pre-incubation with MEA, for if the culture had been in the growth phase the chemical should have brought it to stationary phase, altering the dose response (Ginsberg, 1966). The development of the shoulder would thus appear to be the result of metabolic changes in the bacteria following the first irradiation, rather than the cessation of division.

When the second radiation dose was sufficient to decrease survival to a point on the exponential portion of the dose-response curve, the slope of the curves were not significantly different from the single-dose response curve. Thus, once the bacteria have accumulated their maximum amount of sublethal damage, the response as measured by survival is similar to that of previously unirradiated bacteria.

It would appear, therefore, that MEA protects the bacteria against the lethal effects of γ-irradiation, and this protection also alters the type of damage occurring in such a way that division is greatly inhibited, and the bacteria can withstand comparatively large amounts of radiation damage before the lethal amount of damage is reached.

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Agglutinating Antigens of *Lactobacillus jugurti* ATCC 521

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SUMMARY

Serological studies were conducted on *Lactobacillus jugurti* (ATCC 521) in an attempt to identify its agglutinating antigens.

Glycerol teichoic acid was isolated from the cell wall of ATCC 521. Isolated wall teichoic acid removed I21 and I22 antibodies from ATCC 521 antiserum, thereby demonstrating that it is an antigen involved in agglutination. I27 antibodies were not removed from antisera by the wall teichoic acid. Isolated ATCC 521 cell walls did not agglutinate in anti-I12 antiserum, nor did wall teichoic acid from the same organism react with this serum.

Intracellular teichoic acid of ATCC 521 also removed agglutinating antibody from whole antisera. Gel double-diffusion experiments indicated that cell wall and intracellular teichoic acid are immunologically identical.

Lactobacillus jugurti (ATCC 521) mucopeptide was prepared from the cell walls, and this component did not remove agglutinating antibody from antisera.

INTRODUCTION

Williams (1948), the first to study the agglutinating antigens of *Lactobacillus casei* and *Lactobacillus plantarum* by agglutinin adsorption analysis, detected five agglutinating antigens, which were lettered from A to E. Additional agglutinating antigens of *L. casei* and *L. plantarum*, designated as F, G, H and I, were detected by Orland (1950). Miller (1957) detected a K and an L antigen in strains of *L. lactis*. The antigenic structure of *L. bulgaricus*, *L. helveticus* and *L. jugurti* was investigated by Mageau (1966) by agglutinin adsorption analysis; he found that *L. helveticus* ATCC 10797 contained an I20 and an I21 antigen. Mageau (1966) also detected in *L. bulgaricus* ATCC 11842, 15062 and 15065 an I21 antigen and a new agglutinogen, I22. The I12 antigen, one previously detected by Efthymiou & Hansen (1962) in *L. acidophilus*, was also observed in ATCC 11842, 15062 and 15065. The I20 and I21 antigens (see footnote to Table 1) have also been observed in an additional strain of *L. helveticus* (ATCC 12046) (personal observation). The I12, I21, I22 and a newly discovered agglutinogen, the I27 antigen, have been observed in *L. jugurti* ATCC 521 (personal observation, see Table 1). In the published investigations mentioned above, no attempts were made to identify the agglutinating antigens.

Baddiley & Davison (1961) found that *Lactobacillus jugurti* NCIB 2889 (ATCC 521) possessed both wall and intracellular teichoic acid. The intracellular teichoic acid has been described as the *L. helveticus-jugurti* serological group A antigen by Sharpe, Davison & Baddiley (1964), but they found that cell-wall teichoic acid from these species did not react with group A antiserum. They did not, however, study the role of the latter acid in the agglutination reaction. Juergens, Sanderson & Strominger (1963) and Nathenson & Strominger (1962) found, however, that the precipitinogen

ribitol teichoic acid, when added to homologous antiserum, inhibited cell-wall agglutinogen of *Staphylococcus aureus*. This evidence indicates that wall teichoic acid may play a role in the agglutination reaction.

Another component of the bacterial cell wall, the mucopeptide, has been studied extensively. Much of the research performed on bacterial mucopeptide has dealt with the elucidation of its structure (Strominger, Izaki, Mutsuhashi & Tipper, 1967; Plapp, Schleifer & Kandler, 1967). Karakawa & Krause (1966) found streptococcal mucopeptide to be immunogenic, but no studies were conducted to determine the role of the mucopeptide in the agglutination reaction. Morse (1962) however found that mucopeptide of *Staphylococcus aureus* did not absorb agglutinating antibody from antiserum.

Table 1. *Antigenic composition of lactobacilli determined by agglutinin-adsorption analysis*

Antisera	Antigens*				
	I12	I20	I21	I22	I27
L. acidophilus, ATCC 4356‡	+	−	−	−	−
L. bulgaricus, ATCC 11842†	+	−	+	+	−
L. bulgaricus, ATCC 15062†	+	−	+	+	−
L. bulgaricus, ATCC 15065†	+	−	+	+	−
L. helveticus, ATCC 10797†	−	+	+	−	−
L. helveticus, ATCC 12046§	−	+	+	−	−
L. jugurti, ATCC 521§	+	−	+	+	+

* Williams (1948) used letters to designate the antigens in lactobacilli. Williams *et al.* (1953) later suggested the use of a system involving numbers to replace the letters, i.e. A = I1, B = I2, etc., since the alphabet may not contain a sufficient number of letters to accommodate all of the antigens.

† Mageau (1966). ‡ Efthymiou & Hansen (1962). § Personal observation.

Foley & Wheeler (1945) detected four types of agglutinating antigens within the group D streptococci and found that this was in agreement with the number detected by the precipitin reaction. Sharpe & Shattock (1952) found in typing group D streptococci that there appeared to be some correlation between the precipitin and the agglutination reactions. Lancefield (1943) observed that the M antigen in group A streptococci could be detected by agglutination and precipitin tests. No studies were performed, in the cases cited, to determine whether agglutination and precipitin reactions are caused by identical antigens.

In view of the findings of others, studies were conducted to determine the roles of the cell-wall teichoic acid and the mucopeptide of *Lactobacillus jugurti* ATCC 521 in the agglutination reaction and the composition of the I12, I21, I22 and I27 antigens. Studies were also made to ascertain whether the agglutinogens for *L. jugurti* (ATCC 521) are also precipitinogens and whether there is an immunological similarity between the wall and intracellular teichoic acids.

METHODS

Organisms and conditions of growth. The strains used in these studies were *Lactobacillus bulgaricus* ATCC 11842, ATCC 1506c, ATCC 15062, ATCC 15064, ATCC 15065, L 933 and L 934; *L. helveticus* ATCC 10797, ATCC 10386 and ATCC 12046; *L. jugurti* ATCC 521, ATCC 15063, L 801 and L 803; and *L. acidophilus* ATCC 4356. The L strains

were obtained from Dr P. A. Hansen of the University of Maryland, College Park, Maryland. MRS medium (DeMan, Rogosa & Sharpe, 1960) was used for growing the organisms, which were harvested after incubation at 37° for 18 to 24 hr.

Chemical analysis. For identification of monosaccharides, materials were hydrolyzed for 2 hr at 100° in 2N-H_2SO_4. The residue was dissolved in water and chromatographed with ethyl acetate + methanol + acetic acid + water (80 + 10 + 10 + 15 v/v) by thin-layer chromatography with the Eastman Chromagram System (supplied by Fisher Scientific, Inc.). In the determination of amino acids, materials were hydrolysed for 20 hr at 100° in 6N-HCl with evaporation to dryness. The residue was taken up in an ethanolic solution of 0·5M-HCl and chromatographed in butanol + acetic acid + water (80 + 20 + 20 v/v) or propanal + water (70 + 30 v/v). After the chromatography, amino acids were detected with the ninhydrin reagent, carbohydrates with aniline hydrogen phthalate and phosphoric esters by the method of Wade & Morgan (1953). Protein determinations were performed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of teichoic acid. Cell-wall teichoic acid was prepared by the method of Sharpe *et al.* (1964) by extractions of cell walls with 10% trichloroacetic acid (TCA). Intracellular teichoic acid was prepared by the extraction of freeze-dried cytoplasmic material with 10 % TCA; the cytoplasmic material was obtained by centrifuging down the cell walls and combining the supernatant with the cell-wall washings. The intracellular teichoic acid was further purified by ethanol precipitation.

The mucopeptide portion of the cell wall was prepared by the method of Plapp & Kandler (1967).

Serological methods. Immune sera were prepared by injecting rabbits 12 times over a period of 3 weeks with bacterial suspensions. Five days after the last injections the rabbits were bled.

The tube-agglutination test was performed by the double-dilution method of Williams, Norris & Gyorgy (1953). The final dilution range was between 1/20 and 1/10,240 and the final volume in any one tube was 0·4 ml., 0·2 ml. being diluted serum and 0·2 ml. the antigen suspension. In those cases in which only small amounts of antisera were available, the above procedure was utilized, but the total volume was 0·04 ml., using equal volumes of antigen and antisera.

The antigen + antiserum system was incubated at 37° for 2 hr and refrigerated at 4° overnight. The tubes were read the following day, and the titre recorded as the highest dilution showing agglutination. The control was normal rabbit serum and antigen.

Adsorption of agglutinins with intact organisms was performed by the method of Efthymiou & Hansen (1962). Adsorption of antibody with soluble antigen was accomplished by diluting the antiserum with the latter until the required dilution was obtained. Following this procedure, the system was incubated at 37° and 4°, as described above, after which it was centrifuged to remove all precipitins.

Ring precipitin test. The method of Jones & Shattock (1960) was used. The concentration of teichoic acid in this test was 1 mg./ml. The concentration of teichoic acid in the agar-diffusion test of Ouchterlony (1953) was 1 mg./ml.

Preparation of cell walls. Bacteria previously freeze dried were broken in the French pressure cell at 12,560 lbs/sq. in., after which the material was centrifuged at 480 × G.R.C.F. in a Sorvall Super Speed RC-2 refrigerated centrifuge. The unbroken bacteria were deposited, and the walls were recovered in the supernatant and washed nine

times with M-phosphate buffer, pH 7, followed by an equal number of washings with distilled water. During the washing the walls were exposed to ultrasonic vibrations for 20 sec. at a setting of 2 W. on a Sonifier Cell Disrupter (Heat Systems Co., Melville, Long Island, New York). This procedure was carried out in the presence of glass beads, 1/2 mm. diam., in order to break cell-wall masses. After each washing the protein content of the supernatant was determined by the method of Lowry *et al.* (1951). When little or no protein was found in the washings of the cell walls, the preparation was judged clean. The purity of the cell-wall suspensions was further confirmed by phase microscopy.

Table 2. *Agglutination of Lactobacillus jugurti ATCC 521 and walls with whole and adsorbed antisera*

Antisera	ATCC 521 cells in unadsorbed antisera	Freeze dried ATCC 521 cells in unadsorbed antisera	ATCC 521 cell walls in unadsorbed antisera	ATCC 521 cells in antisera adsorbed with 521 cells	ATCC 521 cells in antisera adsorbed with 521 cell walls	Homologous cells in untreated antisera
L. acidophilus, ATCC 4356	160	160	—	—	—	1280
L. bulgaricus, L 933	80	80	40	—	—	640
L. bulgaricus, L 934	160	320	80	—	—	1280
L. bulgaricus, ATCC 11842	320	320	320	—	—	640
L. bulgaricus, ATCC 15060	160	160	160	—	—	640
L. bulgaricus, ATCC 15062	1280	640	320	—	—	1280
L. bulgaricus, ATCC 15064	320	160	160	—	—	5120
L. bulgaricus, ATCC 15065	320	40	160	—	—	80
L. helveticus, ATCC 10386	640	320	320	—	—	2560
L. helveticus, ATCC 10797	320	320	320	—	—	1280
L. helveticus, ATCC 12046	320	160	160	—	—	640
L. jugurti, ATCC 521	1280	1280	1280	—	—	1280
L. jugurti, L 801	1280	1280	1280	—	—	1280
L. jugurti, L 803	5120	1280	5120	—	—	5120
L. jugurti, ATCC 15063	40	40	40	—	—	640

See footnote to Table 3

RESULTS

Location of the bacterial agglutinating antigens. In order to determine the role of the cell wall in the agglutination reaction, the following experiment was performed. Agglutination tests were conducted with antisera prepared against strains of *Lactobacillus bulgaricus* (ATCC 11842, ATCC 15060, ATCC 15062, ATCC 15064, ATCC 15065, L 933 and L 934), *L. jugurti* (ATCC 521, ATCC 15063, L 801 and L 803), *L. helveticus* (ATCC 10386, ATCC 10797 and ATCC 12046) and *L. acidophilus* (ATCC 4356), all known to agglutinate *L. jugurti* ATCC 521 (see Table 2). Simultaneous agglutination tests were performed

with freeze-dried organisms from which the cell walls were prepared in order to determine whether the antigenic properties of the cells had been impaired during the freeze-drying process. Agglutination tests were also performed with the previously mentioned antisera adsorbed with ATCC 521 or with its corresponding cell walls. These tests indicated that the bacterial cell wall contained the agglutinating antigens.

Role of the cell-wall teichoic acid in the agglutination reaction and its immunologic similarity to intracellular teichoic acid

Having demonstrated that the cell wall contained the antigens that were responsible for agglutination, experiments were made to determine the role of wall and intracellular teichoic acid in the reaction. Chemical analysis of the isolated acids revealed that the wall acid contained glycerol phosphate, alanine and variable traces of glucose. On the other hand the intracellular teichoic acid contained, in addition to these same components, a trace of ribose.

Two mg. of wall teichoic acid and the same amount of intracellular teichoic acid of ATCC 521 were added to separate 0·05 ml. quantities of homologous, whole antiserum to remove the corresponding antibody. The adsorbed serum was then used to perform agglutination tests with various species of lactobacilli (Table 3). *Lactobacillus helveticus* 10797 and 12046, shown in Table 3, contain the I21 antigen and are antigenically identical. *Lactobacillus bulgaricus* 11842, 15062 and 15065 contain the I12, I21 and I22 antigens. An examination of the table indicates that both wall and intracellular teichoic acid absorbed agglutinating antibody from antisera, thereby suggesting that these two substances may be immunologically identical. To investigate this possibility an Ouchterlony double-diffusion test was performed. The line of identification connecting the intracellular and wall teichoic acids indicated that these substances were antigenically identical.

Table 3. *Agglutination of Lactobacillus bulgaricus, L. helveticus, and L. jugurti with Lactobacillus jugurti ATCC 521 antiserum adsorbed with wall and intracellular teichoic acid*

Antigen	ATCC 521 antiserum	ATCC 521 antiserum adsorbed with wall teichoic acid	ATCC 521 antiserum adsorbed with intracellular teichoic acid
L. bulgaricus, ATCC 11842	320*	160	160
L. bulgaricus, ATCC 15062	1280	320	320
L. bulgaricus, ATCC 15065	320	80	160
L. helveticus, ATCC 10797	320	—	40
L. helveticus, ATCC 12046	320	—	—
L. jugurti, ATCC 521	1280	320	640

* Numerical values indicate the denominator of the reciprocal agglutination titre; — no agglutination.

To substantiate these findings further, ATCC 521 antiserum was adsorbed with homologous cell walls after which a gel-diffusion plate was prepared (Fig. 1). This absorbed serum no longer reacted with wall and intracellular teichoic acid. Furthermore, it no longer agglutinated intact homologous cells.

Because absorption of whole ATCC 521 antiserum with intracellular and wall teichoic

acid reduced the agglutination titre in six strains of lactobacilli tested (Table 3), the indication was that these strains contained immunologically similar or identical teichoic acids. To explore this possibility an Ouchterlony double-diffusion test was performed with antisera to ATCC 11842, 15062, 15065, 10797, 12046 and 521 and with wall teichoic acid from the latter organism (Fig. 2). Examination of the Ouchterlony plate revealed that five of the antisera tested reacted with ATCC 521 wall teichoic acid, although the reaction with anti-15062 was very faint. A different sample of ATCC 11842 antiserum, with a low titre of 1/80, was used in the gel-diffusion plate from that utilized in the tests reported in Table 2. It did not react with wall teichoic acid from ATCC 521 in the Ouchterlony plate; however this antiserum did react with the teichoic acid in the ring precipitin test.

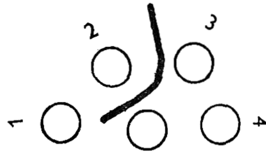

Fig. 1

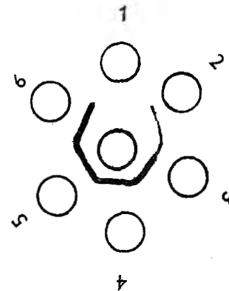

Fig. 2

Fig. 1. Ouchterlony double-diffusion plate. Well 1: normal rabbit serum, well 2: ATCC 521 antiserum; well 3: wall teichoic acid; well 4: ATCC 521 antiserum adsorbed with homologous cell walls; centre well: intracellular teichoic acid.

Fig. 2. Ouchterlony double-diffusion plate. Well 1: 11842 antiserum; well 2: 15062 antiserum; well 3: 15065 antiserum; well 4: 10797 antiserum; well 5: 12046 antiserum; well 6: 521 antiserum; centre well: wall teichoic acid.

The previous experiments indicated that wall teichoic acid is an important antigen in agglutination. Therefore an experiment was designed to determine whether this component was the only or the major antigen responsible for agglutination. Increments of wall teichoic acid of ATCC 521 were added to whole homologous antiserum until no more precipitate formed. Completeness of precipitation was substantiated by finding the antigen in the supernatant. When all of the teichoic acid antibody was removed from the antiserum, the antiserum still exhibited a titre of 1/40, indicating that other factors are involved in agglutination. This absorbed antiserum no longer reacted with ATCC 10797 and 12046, which contain the I21 antigen. Neither did it react with ATCC 11842, 15062 and 15065, which contained the I21 and I22 antigens. The serum, however, agglutinated the homologous organism at a very low titre apparently because of the I27 antigen.

Role of mucopeptide in agglutination. ATCC 521 antiserum was absorbed with the corresponding mucopeptide, after which the former was used for an agglutination test. The titre of the adsorbed serum was identical with that of the unabsorbed serum. Antiserum prepared against mucopeptide also did not agglutinate intact, untreated cells of ATCC 521; however, agglutination was observed with homologous anti-cell-wall serum.

Nature of the I12 antigen. It was noted (Table 2) that anti-I12 serum prepared from

ATCC 4356 did not agglutinate cell walls of ATCC 521. Since evidence from previous experiments indicated that some of the precipitinogens found in the lactobacilli studied were also agglutinogens, experiments were undertaken to determine whether or not this was true of the I12 antigen. Utilization of the precipitin and double-diffusion gel tests revealed that the teichoic acid of ATCC 521 did not react with anti-4356 serum. It was also found that extracts of ATCC 4356 did not react with 521 serum. It was further observed that 521 antiserum did not agglutinate ATCC 4356.

DISCUSSION

As previously mentioned, Morse (1962) found that the mucopeptide of *Staphylococcus aureus* did not adsorb agglutinating antibody from antiserum. This also was found to be true with the mucopeptide of *Lactobacillus jugurti* ATCC 521. However, when working with bacterial mucopeptide prepared by formamide extraction it should be remembered that alteration may occur during the isolation process. Such alteration would result in a component dissimilar to and possessing different immunological properties from those of the native mucopeptide. Perkins (1965) found that formylation of the free amino groups by hot formamide occurred during preparation of the mucopeptide from cell walls.

Evidence presented here indicates that cell-wall and intracellular teichoic acid are immunologically identical, thereby disagreeing with the findings of Sharpe *et al.* (1964). These authors found that wall teichoic acid of ATCC 521 (NCIB 2889) did not react with group antisera. Perhaps the differences are due to the methods by which the antisera were prepared. Antisera used in the present studies were obtained after immunizing each animal for 3 weeks with approximately 12 injections and a total of 14·1 ml. of antigen. A schedule of this nature produces an antiserum with a very high titre. There is the possibility that the antiserum utilized by Sharpe *et al.* (1964) was of a much lower titre, the reaction with wall teichoic acid thereby not being discernible. There is also the possibility that the medium in which the organisms were grown might be responsible for the differences in wall teichoic acid as detected in these studies and as reported by Sharpe *et al.* (1964). This possibility should be considered in light of the findings of Ellwood & Tempest (1967) and Tempest, Dick & Ellwood (1968), who found that the teichoic acid prepared from *Bacillus subtilis* grown in a magnesium-limited medium contained glycerol, glycerol phosphate and glucose. When this organism was grown in a phosphate-limited environment, the wall teichoic acid, upon hydrolysis, showed the absence of glycerol, glycerol phosphate and glucose but the presence of uronic acid and an amino sugar, indicating a teichuronic acid-type compound. Ellwood & Tempest (1968) also found that the cell walls of *Bacillus subtilis* W 23, which normally contain ribitol teichoic acid, manifested a teichuronic acid polymer when the organism was grown in a phosphate-limited medium. Baddiley (1968) also reported that the number of glucosyl substituents on the ribitol teichoic acid from the walls of *Lactobacillus arabinosus* 17-5 depended partly upon the concentration of glucose in the growth medium.

Sharpe *et al.* (1964) observed that *Lactobacillus helveticus* and *L. jugurti* contained wall glycerol teichoic acid. If this is true of all representatives of these species, then it can be assumed that ATCC 10797 contains the same kind of polymer. *Lactobacillus helveticus* ATCC 10797 and *L. jugurti* ATCC 521, although containing wall glycerol teichoic acid, manifest differences in agglutinating antigens. *Lactobacillus helveticus* 10797

contains the I20 and I21 antigens (Mageau, 1966), and *L. jugurti* 521 the I12, I21, I22 and I27 antigens. Since it appears that the main agglutinating antigen in the species discussed is wall glycerol teichoic acid, these antigenic dissimilarities are probably due to differences in this substance.

ATCC 10797 and 521 antisera exhibited a reaction of identity when challenged with *L. jugurti* ATCC 521 wall teichoic acid. Because teichoic acid removes the anti-I21 and anti-I22 components from homologous antiserum, one is led to suspect that two separate and different acids exist in the wall of 521, one the I21 and the other the I22 antigen. In reference to this last speculation, *Staphylococcus aureus* H was found by Baddiley, Buchanan, Martin & Rajbhandary (1962) to contain a wall ribitol teichoic acid substituted with alanine and *N*-acetyl-glucosamine in α- and β-glycosyl linkages. It was further found that the α and β anomers occurred on different chains. Although the possibility of 521 walls containing two types of teichoic acid exists, it was not borne out by gel-diffusion tests. If two different types of teichoic acid were present in the walls of ATCC 521, one would expect to observe two lines in a gel-diffusion test when challenged with homologous serum, but this was not seen. A phenomenon similar to this was observed by Torii, Kabat & Bezer (1964): they demonstrated that *S. aureus* walls contained two types of ribitol teichoic acid, one with α-*N*-acetyl-glucosaminyl and the second with β-*N*-acetyl-glucosaminyl linkages. They were able to separate these two polymers and to demonstrate that they were different immunologically. Attempts to demonstrate two lines in a gel-diffusion system with homologous antiserum resulted in failure.

Although the wall and intracellular teichoic acid appear to be immunologically identical, it is doubtful whether the latter plays a role in the agglutination reaction. The fact that the agglutinating antibody can be removed from antisera with cell walls substantiates the conclusion that the intracellular teichoic acid plays no part in bacterial agglutination. Furthermore, agglutination of intact cells via intracellular teichoic acid would be unlikely because this involves passage of antibody across the cell wall.

The nature of the I12 antigen detected in *Lactobacillus jugurti* ATCC 521 could not be determined. An examination of Table 2 reveals that isolated cell walls of ATCC 521 did not agglutinate in I12 antiserum. There is a distinct possibility that the antigen involved was destroyed during the isolation of the walls. Walls of strains of *L. acidophilus* investigated by Ikawa & Snell (1960) were found to contain glycerol phosphate, a component of teichoic acid. Dr O. Kandler (personal communication) found teichoic acid in the cell walls of strains of *L. acidophilus* investigated. Morse (1963) observed that dilute acid extracts of *Staphylococcus albus* contained wall teichoic acid. In the present study it was found that acid extracts of *L. acidophilus* ATCC 4356, which should contain wall teichoic acid, did not react with ATCC 521 antiserum. This preliminary experiment, along with the fact that ATCC 4356 (I12) antiserum did not react with ATCC 521 teichoic acid, indicates that immunologically dissimilar acids are found in the cell walls of these two species. It also demonstrates that ATCC 521 wall teichoic acid is probably not the I12 antigen.

Experiments revealed that wall teichoic acid did not remove all of the agglutinating antibody from homologous antiserum. This might indicate either that another component might be responsible for a portion of bacterial agglutination or that the teichoic acid or mucopeptide was altered to such an extent during the isolation process that its antigenicity was impaired.

Some mention should be made of the serological cross reactions between strains of *Lactobacillus helveticus*, *L. jugurti* and *L. bulgaricus*. Tables 1 and 2 indicate a close immunological relationship between the species investigated. *L. jugurt* does not seem to be any closer to *L. helveticus* antigenically than to *L. bulgaricus*. The close serological relationships between *L. helveticus* and *L. jugurti* correlates with the similarity in their physiological characterization established by Orla-Jensen (1919). These organisms are so similar physiologically that it is hardly justified to recognize two species (Hansen, 1965). The serological studies performed demonstrate that *L.bulgaricus* is quite close to *L. helveticus* and *L. jugurti* antigenically, but it is well known that the former is quite distinct physiologically from the latter two (Hansen, 1965).

It has been known for some time that serological relationships exist between some lactobacilli which are physiologically dissimilar. Miller (1957) observed that various strains of *L. lactis* contained I1, I2 and I3 and other agglutinating antigens formerly detected only in *L. casei* and *L. plantarum*. Efthymiou & Hansen (1962) found agglutinating antigens previously observed in *L. casei*, *L. plantarum* and *L. lactis* present in strains of *L. acidophilus*. Mageau (1966) found agglutinating antigens previously observed in *L. casei*, *L. plantarum*, *L. lactis* and *L. acidophilus* present in *L. jugurti* and *L. bulgaricus*. To complicate the situation further Chung & Hawirko (1962) found that *L. casei* and *L. lactis* shared agglutinating antigens with *Streptococcus lactis* and *Streptococcus cremoris*. These facts substantiate the observation made in this study that the sharing of agglutinating antigens among different species of lactobacilli observed in this study is not an unusual phenomenon.

One important aspect of the studies conducted is the fact that the agglutinating antigens can be detected via the precipitin test with pure wall teichoic acid. This should prove useful for the identification of species of the genus *Lactobacillus* and should be of value in the elucidation of various taxonomic difficulties.

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Genetic Heterology between *Escherichia coli* K12 and a Smooth Strain of *E. coli*

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SUMMARY

CF2004-6, a recipient strain, and 04M2, an Hfr donor strain of *Escherichia coli* 04:K(undetermined):H5, were used in mating experiments with *E. coli* K12. 04M2 behaved like the K12 donor strain in mating experiments. These donor strains genetically recombined with the K12 recipient at higher frequencies than with CF2004-6. The results of interrupted mating experiments were also more typical with the K12 recipient than with CF2004-6 in that there were sharp entry times for the markers studied and linear increases of recombinants with time. The possibility that host-controlled restriction decreased genetic recombination between *E. coli* K12 and CF2004-6 was pursued. We obtained no evidence for this possibility. Instead the data suggest that genetic heterology between these strains is affecting recombination. Genetic heterology also explains why 04M2 behaves more like a K12 donor in mating experiments with CF2004-6.

INTRODUCTION

Smooth strains of *Escherichia coli*, i.e. those which are known to contain O and K antigens, can be sexually competent (Ørskov & Ørskov, 1961, 1966; Ketyi & Szendrei, 1967). Many if not the majority of the freshly isolated strains used in early fertility surveys (Lederberg, Cavalli & Lederberg, 1952; Furness & Rowley, 1957) and more recently for transfer of a variety of plasmids (Meynell, Meynell & Datta, 1968) were probably smooth. However, most of these strains were used only to establish donor or recipient ability or to study the transfer of a limited number of markers attached to the plasmids. When CF2004-6, a strain of *E. coli* 04:K(undetermined):H5, was shown to be a recipient (Zubrzycki & Spaulding, 1965) it became of interest to see how this typical smooth strain behaved in mating experiments. We found that it did not mate in broth, possibly because surface antigens prevented effective contact formation. The Millipore filter technique described in the Methods section allowed us to use CF2004-6 in kinetic mating experiments. In preliminary work we became convinced that the genetic map of our smooth strain of *E. coli* would be similar to that established with K12 (Taylor & Trotter, 1967). We did find that the recombination frequencies in *E. coli* K12 × CF2004-6 matings were lower than those in K12 × K12 matings. Because host-controlled restriction is the cause of low recombination frequencies between *E. coli* K12, and *E. coli* B strains (Boyer, 1964; Copeland & Bryson, 1966) and *E. coli* K12 (P1) (Arber & Morse, 1965; Colson, Glover, Symonds & Stacey, 1965), we decided to check whether it occurred in our studies. The data presented in this paper suggest that genetic heterology (some type of dissimilarity between DNA which

hinders recombination) rather than restriction accounts for the low recombination frequencies between *E. coli* K12 and CF2004–6 strains.

METHODS

Media. Penassay broth (antibiotic medium No. 3, Difco) was used for the cultivation of the organisms. The minimal agar was essentially the Davis minimal medium prepared as described by Lederberg (1950) except that it contained 0·5% glucose and approximately 1·3% Noble agar (Difco). The amino acids were used at a concentration of 30 to 40 μg./ml. Streptomycin was used at a concentration of 500 μg./ml. to counterselect against the donor strains. Minimal enriched agar was minimal agar enriched with 0·2% Penassay broth.

Bacteria. Our recipient smooth strain of *Escherichia coli* is labelled CF2004–6. Mutants of CF2004–6 were isolated after treating a 24 hr culture with 0·1 M-ethyl methane sulfonate for 1 hr. An Hfr donor strain, 04M2, of CF2004–6 was obtained by selection for a terminal marker, lactose, in a mating between P4X6 and Lac 6. All these strains plus the K12 strains are listed in Table 1.

Table 1. *Strains of Escherichia coli used*

Strain designation	Strain origin	Characteristics*	Sex
LZ–TM–6	CF 2004–6	*trp, leu, arg, his, pro, str-r*	F^-
LZ–TM–9	CF 2004–6	*trp, leu, arg, his, thr, pro, str-r*	F^-
Lac 6	CF 2004–6	*trp, ilv, lac*	F^-
04M2	CF 2004–6	*trp, ilv*	Hfr
P4X6	K12	*met*	Hfr
HfrH	K12	wild type	Hfr
AB1133	K12	*thr, leu, arg, his, pro, thi mal, gal, lac, mtl, xyl, str-r*	F^-

* In the text, reference will also be made to the host-controlled restriction and modification locus which is designated as *hsp* (Taylor & Trotter, 1967).

The order of transfer of markers from P4X6 and 04M2 in a counterclockwise direction is *0-pro-leu-thr-arg-his...lac*. The order of transfer of markers from HfrH in a clockwise direction is *0-hsp-thr-leu-pro-his...arg*.

Millipore filter matings. CF2004–6 strains do not conjugate well in broth. We therefore modified the Millipore mating technique described by Matney & Achenbach (1962) and Sanderson (1967). Overnight cultures of donor and recipient were diluted 1/100 in Penassay broth. The female culture was incubated in a 37° water bath shaker for 3 hr and reached 5 to 8×10^8 viable bacteria per ml. The donor culture was incubated for 3 hr in a 37° water bath without shaking and reached 3 to 5×10^8/ml. Nine ml. of the female culture and 1 ml. of the male culture were mixed. The female–male mixture was kept in an ice bath to prevent growth and mating. One ml. samples were filtered on to 27 mm. Millipore filters (type HA). The filter was transferred to 2 ml. of minimal glucose broth in a tube standing in a 37° water bath. Timing started at this point. All matings were terminated by removing the tubes from the water bath, adding 8 ml. of ice-cold saline and agitating the tube on a Vortex Jun. mixer (maximum speed) for approximately 80 sec. Appropriate dilutions were made in saline and 0·1 ml. samples were placed into Petri dishes. Melted minimal agar kept at approximately 50° was poured into each dish and mixed

thoroughly with the 0·1 ml. samples. After hardening, the agar plates were incubated at 37° for 48 hr. This pour plating technique gave us higher recombinant frequencies and more reproducible results than spreading samples on the surface of agar.

Linkage analyses. Linkage between markers was calculated using the following formula:

$$\frac{\text{no. recombinants for markers a and b}}{\text{no. recombinants for marker a}} \times 100 = \%\ a^{+} \text{ recombinants that are also } b^{+}.$$

One tenth ml. samples of the recombinant mixture were plated in agar lacking one amino acid in order to determine the number of recombinants which received one marker and in agar lacking two amino acids for double recombinants. Susan U. Levinson (unpublished) found this technique gave results comparable to those obtained by selecting approximately 200 colonies and scoring for unselected markers on appropriate minimal agar. The frequencies obtained by this technique should be much more reliable because of the greater numbers involved in the analyses.

Marker stability of Escherichia coli recombinants. Unstable heterogenotes occur among hybrids of enteric bacilli (Falkow, Rownd & Baron, 1962; Luria & Burrous, 1957) and even among recombinants of *Escherichia coli* (Lederberg, 1949; Ketyi & Szendrei, 1967). All recombinants to be used in backcrosses were tested for marker stability by streaking on minimal agar, incubating for 48 hr and then restreaking on minimal enriched agar. Reconstruction experiments using known unstable *E. coli* K12-CF2004-6 recombinants (none of which occurred in this study) showed that they grew out in 48 to 72 hr as a mixture of regular sized and tiny colonies.

RESULTS

In preliminary mating experiments between *Escherichia coli* K12 and CF2004-6 we found *lac*, *pro*, *leu*, *thr*, *arg*, *ilv*, *mal*, *his* and *trp* markers to be located in CF2004-6 where expected, according to the genetic map established with *E. coli* K12 (Taylor & Trotter, 1967). In addition to the linkages reported in detail here, we found *xyl* closely linked to the *str* locus and *ilv* to a rough locus, possibly the *E. coli* equivalent of the *rou A* locus described in *Salmonella typhimurium* (Subbaiah & Stocker, 1964). These results convinced us that the genetic map of our smooth strain of *E. coli* was similar to that of *E. coli* K12. However, genetic recombination in these inter-strain matings occurred at low frequencies. We decided to test whether host-controlled restriction could account for these low frequencies as it undoubtedly does in matings between rough strains of *E. coli*.

In matings involving the K12 donor P4X6 and the CF2004-6 recipient LZ-TM-6, one obtains lower recombinant frequencies and decreased linkage between markers when compared with an intra-strain K12×K12 mating using AB1133 as a recipient, Table 2 (crosses 1 and 2) and Table 3 (Test 1). Since 04M2 is a derivative of CF2004-6, we now expected higher recombinant frequencies and increased linkage between markers when it conjugates with LZ-TM-6. The results (Table 2, crosses 3 and 4) show that except for the arginine marker 04M2 recombines with LZ-TM-6 and AB1133 at frequencies comparable to those using P4X6 (crosses 1 and 2). Explanations for these results will be offered later, but at this time it should be pointed out that the higher recombinant frequency for *arg* indicates that the lower recombinant frequencies for other markers observed with LZ-TM-6 were not due to it being a poor recipient. The

results of linkage analyses similarly show that O4M2 behaved like P4X6 (Table 3, Tests 1 and 2). The exception again was with the *arg* marker of LZ-TM-6 which showed increased linkage to *pro* (from 3·9 to 10%) and *leu* (from 2·4 to 25%) in matings with O4M2. The results of interrupted mating experiments (Fig. 1 to 4) are expressed using different scales because of the range in recombinant frequencies, but this cannot

Table 2. *Recombinant frequencies after 100 min. matings*

Cross	Donor × Recipient	Percent recombinants per input donor		
		Pro⁺	Leu⁺	Arg⁺
1	P4X6 × AB1133	23·0	27·0	0·32
2	P4X6 × LZ-TM-6	0·22	2·6	0·26
3	O4M2 × AB1133	19·0	18·0	0·14
4	O4M2 × LZ-TM-6	0·42	2·8	2·6
5	P4X6 × H3	0·23	4·3	0·25
6	P4X6 × H1	—	3·8	0·27
7	HfrH × AB1133	28·0	48·0	—
8	HfrH × LZ-TM-6	1·3	2·3	—
9	HfrH × H3	6·5	8·3	—
10	HfrH × H1	—	6·6	—
11	P4X6 × H4	8·8	2·9	0·27

The average recombinant frequencies from repeat mating experiments is shown in crosses 1, 2, 3, 4, 7 and 8. Only the average recombinant frequencies from backcrosses with individual K12-CF2004-6 hybrids is shown in crosses 5, 6, 9, 10 and 11 because the individual frequencies varied no more than those observed in repeat mating experiments using the Millipore technique as described in the Methods section.

Table 3. *Linkage from 100 min. inter- and intra-strain matings*

Donor	Test	Recipient					
		Pro		Leu		Arg	
		AB1133	LZ-TM-6	AB1133	LZ-TM-6	AB1133	LZ-TM-6
P4X6	1	100·0	100·0	48·0	31·0	0	3·9
		58·0	4·3	100·0	100·0	1·0	2·4
		30·0	2·8	37·0	12·0	100·0	100·0
O4M2	2	100·0	100·0	45·0	32·0	0	10·0
		62·0	6·3	100·0	100·0	1·0	25·0
		33·0	1·8	34·0	16·0	100·0	100·0
HfrH	3	100·0	100·0	83·0	53·0	—	—
		67·0	47·0	100·0	100·0	—	—

Only the average per cent linkages from backcrosses with different hybrids is shown because the individual results did not vary much from the average. The per cent linkage between two markers was determined by comparing the number of recombinants which received the two markers to those which received one of the markers which is expressed in the table as 100%. This was done after a 100 min. mating by plating aliquots of the recombinant mixture in agar lacking two amino acids in order to determine the number of double recombinants and in agar lacking one amino acid for single recombinants.

affect the interpretation of the data which show the appearance of markers at about the same time in each cross. Note the non-linear appearance of markers in Fig. 3 and 4. The non-linear appearance of markers during conjugation was shown to be associated with host-controlled restriction by Copeland & Bryson (1966). However, the same type of curve has even been observed between *Escherichia coli* K12 strains, P4X6 and

AB1133 (Schneider & Falkow, 1964). Therefore, a non-linear curve is no indication that restriction is occurring. The great delay in the appearance of markers observed by Boyer (1964) is an obvious manifestation of restriction, but this did not occur in our crosses. Nevertheless, host-controlled restriction could still account for the lower recombinant frequencies and reduced linkages obtained wtih LZ-TM-6. If this is so,

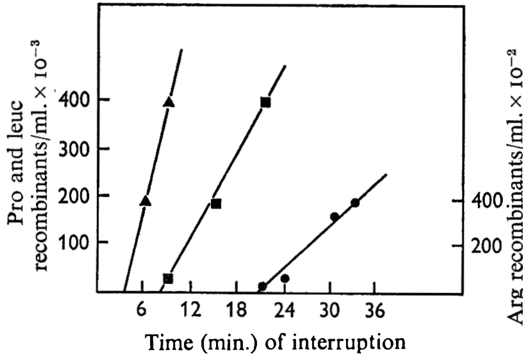

Fig. 1

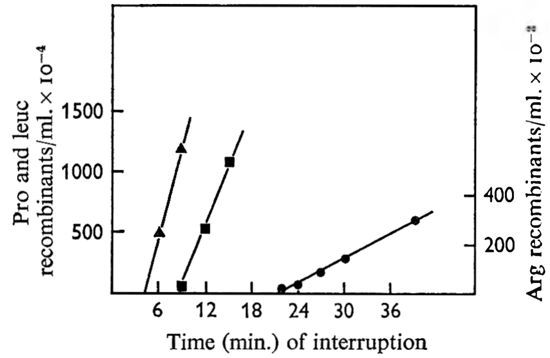

Fig. 2

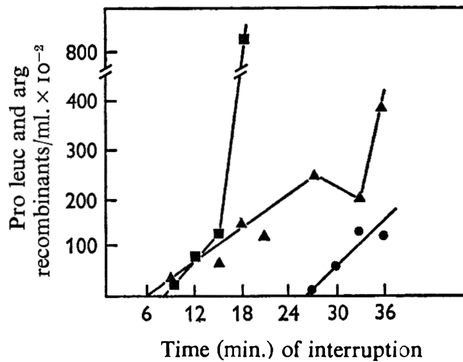

Fig. 3

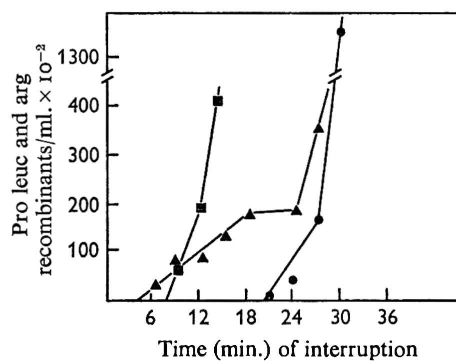

Fig. 4

Fig. 1. Interrupted mating experiment between P4X6 and AB1133. One ml. mixtures of log. phase donor (3 to 5×10^7 ml.) and recipient (5 to 8×10^8/ml.) cultures were filtered on to Millipore filters. The filters were placed in tubes containing minimal glucose broth which were incubated at 37°. At time intervals cold saline was added to the tubes which were then shaken on a Vortex Jun. mixer in order to interrupt the matings. Samples were now plated in order to select for Pro⁺ (▲—▲), Leu⁺ (■—■) and Arg⁺ (●—●) recombinants.

Fig. 2. Interrupted mating experiment between O4M2 and AB1113. Pro⁺ (▲—▲), Leu⁺ (■—■) and Arg⁺ (●—●) recombinants. For details see Fig. 1.

Fig. 3. Interrupted mating experiment between P4X6 and LZ-TM-6. Pro⁺ (▲—▲), Leu⁺ (■—■) and Arg⁺ (●—●) recombinants. For details see Fig. 1.

Fig. 4. Interrupted mating experiment between O4M2 and LZ-TM-6. Pro⁺ (▲—▲), Leu⁺ (■—■) and Arg⁺ (●—●) recombinants. For details see Fig. 1.

then incorporation in a CF2004-6 recipient of the K12 host-controlled restriction and modification locus (K12-*hsp* locus) should make them appear more K12-like in matings. This work was pursued using only K12 donor strains since we knew that restriction and modification occur with K12 strains. The approach we took was similar to that

used by Boyer (1964), who found linkage between the *hsp* locus and the *thr* marker. An HfrH × LZ–TM–9 mating was interrupted at 6, 9 and 12 min. From the various time intervals a total of eighteen Thr$^+$ recombinants (labelled H3) was isolated. The recombinants were checked for stability and then backcrossed with P4X6 (Table 2, cross 5). There was no marked increase in recombinant frequencies for *pro* or *arg* when compared to the results shown in cross 2, but there did seem to be a slight increase for *leu*. Linkage analyses were done on all crosses (Table 4, Test 1 with H3). Compared to the results using LZ–TM–6 (Table 3, Test 1 with LZ–TM–6), there was an increase in linkage for *leu* both among Pro$^+$ (from 31 to 58 %) and Arg$^+$ (from 12 to 26 %) recombinants. That these slight increases for *leu* recombination and linkage may not be specifically related to the K12-*hsp* locus is suggested by the following experi-

Table 4. *Linkage from 100 min. backcrosses with recipient K12–CF2004–6 hybrids*

		Recipient							
		Pro		Leu			Arg		
Donor	Test	H3	H4	H3	H1	H4	H3	H1	H4
P4X6	1	100·0	—	58·0	—	—	2·1	—	—
		2·9	43·0	100·0	—	100·0	1·7	—	1·3
		1·7	19·0	26·0	23·0	20·0	100·0	100·0	100·0
HfrH	2	100·0	—	68·0	—	—	—	—	—
		43·0	—	100·0	—	—	—	—	—

Only the average per cent linkages from backcrosses with different hybrids is shown because the individual results did not vary much from the average. The per cent linkage between two markers was determined by comparing the number of recombinants which received the two markers to those which received one of the markers which is expressed in the table as 100 %. This was done after a 100 min. mating by plating samples of the recombinant mixture in agar lacking two amino acids in order to determine the number of double recombinants and in agar lacking one amino acid for single recombinants.

ments. A P4X6 × LZ–TM–6 conjugation was interrupted at 6 and 9 min. in order to get Pro$^+$ recombinants. Eleven Pro$^+$ recombinants (labelled H1) were isolated, checked for stability, found to be *leu* negative and presumed to be K12-*hsp* negative since this locus is even distal to *leu*. The recombinant frequencies from backcrosses with P4X6 and these recombinants are shown in Table 2. Compared to the results using LZ–TM–6 (cross 2), backcrosses using H1 (cross 6) resulted in a slight increase for Leu$^+$ recombinants similar to that using H3 (cross 5). Since H1 is Pro$^+$, the only linkage determined was that between *arg* and *leu* which is shown in Table 4 (Test 1 with H1). Compared to the results using LZ–TM–6 (Table 3, Test 1 with LZ–TM–6), there was a slight increase in this linkage (from 12 to 23 %) similar to that observed with the H3 recipients (from 12 to 26 %). Another *E. coli* K12 donor strain, HfrH, was used in experiments similar to those just described for P4X6. The recombinant frequencies in inter-strain matings were considerably lower than those in intra-strain matings as shown in Table 2 (crosses 7 and 8). In spite of this, linkage between *pro* and *leu* in the inter-strain matings was not greatly affected (Table 3, Test 3, compare the results using AB1133 and LZ–TM–6). Backcrosses with six H3 recombinants resulted in some increase in recombinant frequencies for *pro* and *leu* (Table 2, compare crosses 8 and 9). Linkage analyses on these backcrosses are shown in Table 4 (Test 2 with H3). Compared to the

results using LZ–TM–6 (Table 3, Test 3 with LZ–TM–6), selection for the distal marker *pro* resulted in an increase in linkage with *leu* (from 53 to 68%). However, when selecting for the proximal marker *leu* there was no increase in linkage. HfrH was also backcrossed with three H1 recombinants. Compared to the results using LZ–TM–6 (Table 2, cross 8), backcrosses using H1 (cross 10) resulted in a slight increase for Leu^+ recombinants similar to that using H3 (cross 9). Since H1 is Pro^+, the recombinant frequency and linkage for this marker could not be determined.

In all these backcrosses using K12–CF2004–6 recombinants slight increases in recombinant frequencies and linkage could be associated with the incorporation of the K12–*thr* marker which is closely linked to the K12-*hsp* locus. But that does not mean that the K12-*hsp* locus is functioning in CF2004–6 because similar increases were also found with recombinants which contained a K12 region (*pro*) not closely linked to the K12-*hsp* locus. Our interpretation for these slight increases is that the presence of a piece of K12 DNA in CF2004–6 increases the chances of crossing over between this K12 DNA and the homologous DNA region in the *E. coli* K12 donor, thus increasing genetic recombination. Take, for example, the results obtained from matings between *E. coli* K12 and H3. The slight increases for Leu^+ recombinant frequency and linkage are probably due to the presence of the adjacent K12 *thr* region in H3. To support this interpretation we present the results of backcrosses with two typical H1 recombinants which were mutated at the K12 *pro* region (labelled H4). Backcrosses involving P4X6 and H4 show a higher Pro^+ recombinant frequency when compared with a mating using LZ–TM–6 (Table 2, crosses 2 and 11). Linkage analyses on these crosses are shown in Table 4 (Test 1 with H4). Compared to the results using LZ–TM–6 (Table 3, Test 1 with LZ–TM–6), there was an increase in linkage for *pro* (from 4·3 to 43%) when selecting for *leu* and increases for *pro* (from 2·8 to 19%) and *leu* (from 12 to 20%) when selecting for *arg*. These results show that areas of DNA homology do increase the chances of recombination between strains of *E. coli*.

DISCUSSION

The data presented in this paper suggest that restriction plays no obvious role in genetic recombination between *Escherichia coli* K12 and CF2004–6 strains for the following reasons. In spite of lower recombinant frequencies and decreased linkage between genes, there was no delay in appearance of markers. Backcrosses with eighteen *E. coli* K12–CF2004–6 recombinants which received the *thr* locus and presumably the K12-*hsp* locus from HfrH showed no more of an increase in recombinant frequencies and linkage than did backcrosses with recombinants which received from P4X6 the *pro* locus, which is not linked to *hsp*.

Boyer (1964) found that the K12-*hsp* locus was incorporated along with the *thr* marker in 60% of the *Escherichia coli* B/R recombinants. It may be argued that this and linkage is greatly reduced in CF2004–6 strains. As shown in Table 3, linkage of *leu pro* in HfrH × CF2004–6 matings was decreased not even twofold when compared with linkage in HfrH × AB1133 matings. This difference is even less than that reported for the same two markers in *E. coli* HfrH × B/R matings (Boyer, 1964). Therefore, it seems likely that the closely linked markers, *hsp* and *thr*, would both be incorporated in CF2004–6 in as high a percentage of cases as they were in *E. coli* B/R.

We attribute the lower recombination frequencies to genetic heterology between

Escherichia coli K12 and CF2004–6 and the increase in these frequencies upon backcrossing with these inter-strain recombinants to the artificially established genetic homology. This interpretation is the same as that invoked by Johnson, Falkow & Baron (1964) to explain the lower recombination frequencies between *E. coli* and *Salmonella typhi* and the increase in frequencies upon rematings with hybrids. Genetic heterology can also be used to explain why *E. coli* 04M2, an Hfr derivative of CF2004–6, gives higher recombination frequencies with the K12 recipient than with CF2004–6. In deriving this strain (see Methods), not only did the *E. coli* K12 *lac* region and the F factor become incorporated into the CF2004–6 genome but possibly the *pro* to *leu* region as well. The *arg* region did not, and that is why there is more genetic recombination for this region between 04M2 and CF2004–6. This hypothesis is supported by experiments (unpublished) in which we use transductions by Φ04–CF to distinguish between the genes of *E. coli* K12 and CF2004–6 (Zubrzycki & Gainsburg, 1966).

During the process of the work reported here, we realized that the restriction mechanism is probably a surface phenomenon (Schell & Glover, 1966). In this regard, it was suggested that the Millipore mating technique, by artificially forcing bacteria together, bypassed the normal surface opposition of donor and recipient and consequently the restriction mechanism. We obtained *E. coli* B/R strains from Boyer and demonstrated that our mating technique would allow the restriction mechanism to be expressed. We also proved with his strains that our laboratory strain of HfrH contained and could successfully transmit the K12-*hsp* locus to *E. coli* B/R. We also entertained the possibility that the surface antigens on our smooth strain may be interfering with a surface phenomenon such as restriction. We isolated four rough strains of LZ–TM–6. In preliminary experiments we did not observe restriction during conjugation. These experiments were discontinued when a complete host-controlled restriction and modification system was discovered in our smooth strains (to be published). This system is accompanied by radical effects on genetic recombination and, restriction and modification of phage Φ04–CF (Zubrzycki, Green & Spaulding, 1966). Preliminary experiments with this *E. coli* 04 restriction and modification system prove that the K–12 *hsp* locus is closely linked to *thr* in matings with CF2004–6. However, in the experiments reported here the K12–*hsp* locus could not function in CF2004–6. It has been speculated that the restriction and modification phenomenon is of benefit to a strain of bacterium because it allows genetic exchange with its own strain but prevents it with another (Wood, 1966). The results presented in our studies suggest that the occurrence of restriction and modification in genetic experiments between bacterial strains is unpredictable and the true evolutionary value of the phenomenon is obscure.

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Genetic Transformation in Rhizobium

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SUMMARY

Mutants of ground nut (*Rhizobium cowpea* group) strain GR3 were isolated using ultraviolet radiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as mutagenic agents. An adenine-requiring mutant was used as the recipient and its wild type as the donor in transformation studies. Initially minimal medium supplemented with 2 μg. adenine/ml. was used for the development of competence. The maximum transformation frequency was obtained after about 120 min. incubation. Supplementation of minimal medium with yeast extract and vitamin-free Casamino acids without the addition of adenine stimulated competence to a large extent. The competence was not further stimulated by any of the divalent ions used. The optimum DNA concentration for maximum transformation frequency was about 50 μg./ml.

INTRODUCTION

Ravin (1961) listed more than 20 bacterial species which have been found to be transformable inter-specifically or intra-specifically. Ravin (1961) also commented on transformation in Rhizobium reported by Balassa (1955, 1957, 1960). The original reports do not however contain detailed accounts of the procedures used.

Since then well-defined methods of genetic transformation have been established in Streptococcus, Diplococcus, Pneumococcus, Haemophilus and various strains of *Bacillus subtilis*. Considerable information is available regarding the competence state of the recipient cell, uptake of DNA, integration of DNA and expression of phenotypic characters. Such studies on a transformation system in Rhizobium would be helpful in several ways; for example, whether the mechanism of transformation in other organisms applies to Rhizobium and also whether transformation could be used to study the mechanism of the nitrogen fixation, elucidation of the genetic elements involved and ultimately the exploration of better and more efficient nitrogen fixing strains with a wide host range. More recently studies on genetic transformation between species and genera of various organisms have been extended to establish the taxonomic relationships between them (Marmur, Falkow & Mandel, 1963; De Ley, 1964).

Earlier we confirmed that some strains of Rhizobium are transformable (Gadre, Mazumdar, Modi & Parekh, 1967). Further efforts reported here were made to establish methods for demonstrating genetic transformation in Rhizobium.

METHODS

Strain. The strain GR3 of Rhizobium originally isolated from ground nut host nodules belonging to the cowpea group was kindly supplied by Dr V. P. Bhide, Poona, India. The purity of the culture was tested before use and was maintained on mannitol yeast extract agar medium and subcultured every 15 days.

Media. Complex medium: K_2HPO_4, 0·36%; KH_2PO_4, 0·04%; $MgSO_4.7H_2O$, 0·005%; NaCl, 0·05%; $(NH_4)_2SO_4$, 0·1%; yeast extract (Difco) 0·1%; vitamin-free Casamino acids (Difco) 0·1% and glucose 0·1%. Development of competence was investigated in medium of the following composition: K_2HPO_4, 0·7%; KH_2PO_4, 0·2%; Na-citrate. $2H_2O$, 0·01%; $MgSO_4.7H_2O$, 0·05%; glucose 0·2% and adenine 2 μg./ml. Medium of this composition containing 1·5% (w/v) agar (Difco) without adenine was used for measuring the efficiency of transformation to prototrophy. This competence medium was supplemented with Difco yeast extract and vitamin-free Difco Casamino acids (1 mg./ml. each) for the stimulation of competence. The optimum DNA concentration and effect of divalent ions on competence was investigated using this medium.

Isolation and characterization of mutants. Auxotropic mutants of strain GR3 were isolated using u.v. irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Aldrich Chemicals, U.S.A.) as the mutagenic agents. Initially, u.v. survival curves and NTG inactivation curves were studied. NTG inactivation was studied in acetate buffer 0·2 M at pH 5·0 and in complex medium. An overnight culture was inoculated into fresh complex medium and incubated for 4 hr on a shaker at 30°, centrifuged and the pellet resuspended in acetate buffer and complex medium containing 1 mg. NTG/ml. At regular intervals samples were removed and after appropriate dilutions plated on complex agar medium, incubated for 24 hr at 30° and the colonies were counted. These plates were replicated on to minimal agar plates to estimate the fraction of mutants amongst the survivors. In each case when the survival reached about 0·2% a sample was incubated overnight in complex medium and the penicillin selection methods of Davis (1949) and Lederberg & Zinder (1948) used for the concentration of mutants. The replica plating technique of Lederberg & Lederberg (1952) was used for the characterization of the mutants isolated. A stable adenine-requiring mutant capable of growing optimally at 20 μg. adenine/ml. on agar medium was selected as the recipient.

Preparation of transforming DNA and its estimation. An overnight culture of adenine-independent strain GR3 grown in complex medium was inoculated in fresh medium of the same composition at a cell density of about 1×10^6 cells/ ml. and incubated on a rotary shaker for 8 hr at 30°. The cells were collected by centrifugation at 0 to 4°, washed twice with saline-EDTA (0·15M-NaCl and 0·1 M-EDTA, pH 7·0 ± 0·2) and DNA was extracted by following the method of Marmur (1961) with the modification that after complete lysis of cells Pronase (Calbiochem) 1 mg./ml. of lysate was added and incubated at 37° for 6 hr (Berns & Thomas, 1965). The DNA after isopropanol precipitation was taken up in sterile saline citrate (0·15M-NaCl and 0·015 M-sodium citrate, pH 7·0) and preserved over a layer of chloroform at 0 to 4°. DNA was estimated by the diphenylamine reaction and O.D. was measured at 600 mμ. u.v. absorption of the samples was noted spectrophotometrically at 260 mμ. *Bacillus subtilis* DNA was taken as the standard in both the cases.

Determination of competence. An overnight recipient culture grown in the complex medium was centrifuged and after washing with standard phosphate buffer (pH 7·0) suspended in fresh medium of the same composition to a density of 1×10^6 organisms/ml. and incubated on a rotary shaker at 30° ± 2 for 4 hr. Thereafter the culture was centrifuged, washed twice as before and resuspended again in competence medium. Every 30 min. the culture was removed for studying competence and DNA (25 μg./ml.) was added to make the final volume to 1 ml. Incubation was carried out at 30° ± 2° for 30 min. and the reaction terminated by adding DNase 50 μg./ml. containing 5 mM-$MgCl_2$. The culture was then incubated for 30 min. and after appropriate dilution plated on minimal medium to select for the transformants and on adenine (20 μg./ml.) supplemented medium for the total count. The plates were incubated for 48 hr and the colonies counted. The controls included (1) plating the culture without DNA treatment, (2) recipient treated with recipient DNA, (3) plating of donor DNA alone, (4) addition of DNase before adding DNA and (5) treatment with DNA isolated from an unrelated organism such as *Bacillus subtilis*. No growth was noted after 48 hr of incubation in any of the controls. When the transformation reaction was terminated by DNase after 30 min. of incubation the transformation frequency obtained was 0·04% (Table 1). Competence of the culture was studied in the medium described earlier and for further studies the same medium supplemented with yeast extract (1 mg./ml.) and vitamin-free Casamino acids (1 mg./ml.) was used.

Table 1. *Transformation of adenine-requiring recipient to prototrophy*

Incubation with	No. of colonies on minimal agar	Transformation frequency (%)
Wild-type DNA	4×10^4	0·04
Wild-type DNA treated with DNase	0	—
Recipient DNA	0	—
Bacillus subtilis DNA	0	—

RESULTS

The lethal effect of NTG on the cells in acetate buffer and complex medium is shown in Fig. 1. Residual NTG was removed before plating by serial dilution. The rate of inactivation in complex medium was twice that in buffer and the frequency of mutants increased gradually to a maximum of 20%. The fraction of the mutants amongst the survivors was almost the same in buffer and in complex medium. The ultra-violet survival curve showed that 2·0% survival was obtained within 300 sec. under standard conditions of exposure.

Growth curves

For the transformation experiments the growth curve of the adenine-requiring recipient was measured in complex medium as well as in minimal medium supplemented with adenine 20 μg./ml. The lag period was twice as long in the supplemented medium as in the complex medium (Fig. 2). The culture remained in the log. phase for 6 to 7 hr and thereafter the stationary phase set in. At the end of the log. phase the cultures reached the same optical density in both media. It can be seen that the

phasing of the cultures is slightly different in the two media and this difference might be responsible for the increase in the number of transformants when the competence medium was used.

Competence and age of culture

Figure 3 shows the number of transformants obtained as a function of the age of the culture in minimal medium (competence medium) and medium supplemented with yeast extract and Casamino acids. In both media the culture attains maximum competence between 90 and 180 min. incubation but in supplemented competence medium the number of transformants obtained was higher than in minimal competence

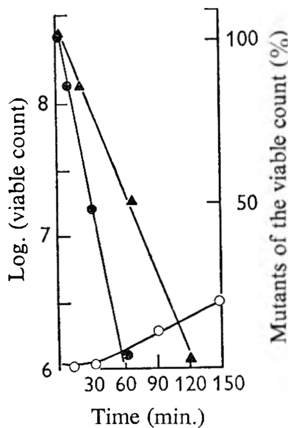

Fig. 1

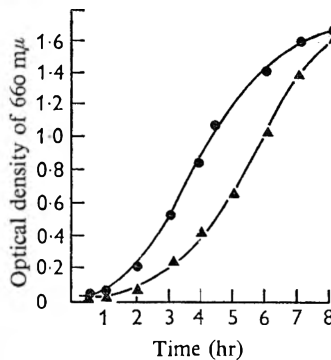

Fig. 2

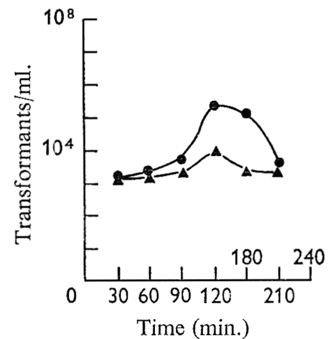

Fig. 3

Fig. 1. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG; 1 mg./ml.) inactivation curve in complex medium (●), acetate buffer (▲) and occurrence of mutants (○) as a function of time.

Fig. 2. Growth curves of the recipient in complex medium (●) and adenine (20 μg./ml.) supplemented minimal medium (▲).

Fig. 3. Competence and age of the culture in minimal medium supplemented with 2 μg. adenine/ml. (▲) and minimal medium supplemented with yeast extract (1 mg./ml.) and vitamin-free Casamino acids (1 mg./ml.) (●). Total count between $2\cdot2 \times 10^8$ and $1\cdot9 \times 10^9$/ml.

medium, indicating that the supplementation causes stimulation of competence. Any further increase in the concentration of yeast extract and Casamino acids interfered with the recovery of the transformants by supporting the growth of non-transformants on the selective minimal agar medium.

Determination of optimum DNA concentration

After 120 min. incubation in the competence medium the culture was removed and treated with various concentration of DNA. The saturating concentration of DNA necessary for maximum transformation was about 50 μg./ml. (Table 2). Increase in the number of transformants was roughly proportional to the amount of DNA added until a concentration of 50 μg./ml. is reached. Concentrations of DNA above 50 μg./ml. decreased the number of transformants slightly.

Effect of divalent ions on competence

Leonard, Mattheis, Mattheis & Housewright (1964) and Young & Spizizen (1963) studied the effect of divalent ions on transformation in *Bacillus licheniformis* and *B. subtilis* respectively. The divalent ions used (Table 3) did not show any stimulation but instead there was a large decrease when Fe was used and a moderate decrease when Cu, Mo, Zn, Mg and Ca were used as compared to the control. The concentrations used were not toxic to the organisms since no effect on the total count of the culture at the time of transformation was observed. Each experimental tube was inoculated with $1 \cdot 5 \times 10^7$ organisms/ml and the total count at the time of transformation ranged between $9 \cdot 8 \times 10^7$ and $1 \cdot 2 \times 10^8$/ml. in all the tubes. We conclude that the transformation process was affected at some stage by the ions used.

Table 2. *Effect of DNA concentration on transformation*

μg. DNA added	Transformants/ml.
0·1	$8 \cdot 6 \times 10^1$
1·0	$1 \cdot 1 \times 10^2$
10·0	$2 \cdot 4 \times 10^2$
20·0	$5 \cdot 0 \times 10^2$
30·0	$8 \cdot 1 \times 10^2$
40·0	$2 \cdot 0 \times 10^3$
50·0	$2 \cdot 2 \times 10^4$
55·0	$2 \cdot 2 \times 10^4$

Total count between 5×10^8 and $6 \cdot 5 \times 10^8$/ml.

Table 3. *Effect of divalent ions on competence*

Ions added*	Transformants/ml.
None	12,000
Cu	100
Zn	110
Mo	170
Mg	190
Fe	30
Ca	50

Total count $9 \cdot 8 \times 10^7 - 1 \cdot 2 \times 10^8$/ml.

* The medium was supplemented with $CuSO_4$, $ZnCl_2$, $(NH_4)\ MoO_2$, $MgSO_4 . 7H_2O$, $FeSO_4$ and $CaCl_2$ (5 μg./ml.) as indicated.

These results show that an adenine-requiring mutant, isolated from the parent strain GR 3, could be transformed by its parent DNA and as is known for some of the other Rhizobium species, competence is essentially attained in the early log. phase. It was necessary to maintain the culture in a good physiological condition to attain maximum transformation frequency and the temperature had to be maintained at $30^\circ \pm 2^\circ$.

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Antigenic Relationships Among Strains of *Mycoplasma mycoides* var. *mycoides*, *M. capri* and *M. laidlawii* Revealed by Complement-fixation Tests

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SUMMARY

Complement-fixation tests were used to compare strains of *Mycoplasma mycoides* var. *mycoides* (5 strains), *M. mycoides* var. *capri* (5 strains) and *M. laidlawii* of bovine genital origin (3 strains). Strains of *M. mycoides* and *M. capri* showed cross-reactions of about 25% identity, but strains of *M. laidlawii* appeared to be antigenically distinct. The results, measured in terms of the amounts of complement fixed, are shown graphically and discussed in relation to the diagnosis of contagious bovine pleuropneumonia.

INTRODUCTION

The clinical diagnosis of contagious bovine pleuropneumonia (CBPP) presents many difficulties because of the occurrence within a herd of atypical cases, subclinical infections and apparently recovered animals which may harbour viable organisms within sequestra in the lungs. Serological tests have been developed to aid diagnosis, notably techniques of agglutination, precipitation, complement-fixation (CF) and allergic skin tests. All have had variable success; none entirely meets the ideal diagnostic requirements of specificity, reliability and ease of use (Gourlay, 1965; Griffin, 1967). The main diagnostic problem remains the recognition of the 'carrier' animal in which the titre of circulating antibody is below the thresholds detectable by existing serological tests. Complement-fixation tests have, in general, been chosen for the examination of sera from individual animals because experience has shown that positive reactions may be expected from the sera of a greater proportion of cattle at different stages of infection than by the use of any other single test. The value of agglutination tests, which for field diagnosis are easier to use, has been limited by lack of specificity and sensitivity manifest in the occurrence of both positive and negative false reactions (Ito, Yamagiwa & Itabashi, 1931; Campbell, 1938; Turner, 1962). An explanation for false negative reactions postulated by Turner was the neutralization *in vivo* of circulating antibody following the release of specific mycoplasma antigen into the circulation, possibly during a septicaemic phase of acute infection. Adler & Etheridge (1964) failed to improve the sensitivity of the test by modification of the method of preparation of the agglutinating suspension. A suggested explanation for false positive reactions is the occurrence of antigens common to *Mycoplasma mycoides* var. *mycoides* (*M. mycoides*) and other mycoplasma species

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or other micro-organisms. The results of CF tests among strains of *M. mycoides*, *M. mycoides* var. *capri* (*M. capri*) and *M. laidlawii* of bovine genital origin are now reported.

METHODS

Complement fixation

A plate complement-fixation (CF) test was used, based on that described by Fulton & Dumbell (1949) in which data obtained from preliminary assays of the reagents were used to establish a comparative test. Differences only from this technique are described.

Test plates. Standard (WHO pattern) plastic haemagglutination plates were used. They were stored in air-tight tins made to hold six plates. The lids of the tins were lined with absorbent paper pads, moistened to maintain a humid atmosphere around the plates during incubation.

Pipettes. Dropping pipettes to deliver 50 drops/ml. were used. Standard graduated glass delivery-pipettes were used to prepare dilutions of all reagents. An automatic syringe of 2·0 ml. capacity calibrated in 0·1 ml. intervals (Bradstreet & Taylor, 1962) was used for the multiple distribution of volumes of reagents.

Glassware for CF tests was not used for other purposes and together with the plastic test plates was washed following the procedure described by Bradstreet & Taylor (1962).

Diluent. Dilutions of the test reagents were prepared in veronal + NaCl buffer (pH 7·3) made up in glass-distilled de-ionized water.

Sensitized sheep erythrocytes. Blood was collected from locally bred sheep kept at the laboratory. With aseptic technique and sterile materials, 100 ml. blood was bled from the jugular vein into 50 ml. modified Alsever solution, thoroughly mixed and allowed to stabilize at 4° for 4 days before use. Thereafter, the blood was stored at 4° up to 28 days provided no discolouration, haemolysis or difficulty in resuspension of the cells after centrifugation during washing was encountered.

A 1% (w/v) suspension of red cells was sensitized by incubation at 37° for 1 hr with an equal volume of horse anti-sheep haemolysin (Burroughs Wellcome Ltd.). A concentration of 4 times the minimal haemolytic dose was used as the optimal sensitizing concentration of haemolysin. This was determined in a preliminary two-dimensional assay of dilutions of haemolysin and guinea-pig complement.

Complement. Guinea-pig serum for complement was collected, pooled, lyophilized in 2·0 ml. volumes and stored at −20°. Ampoules of pooled serum were reconstituted in cooled distilled water and dilutions were made in cooled diluent.

Storage of guinea-pig serum at 4° by Richardson's (1941) method was also tried, but the activity fell appreciably in 6 weeks under conditions in Vom, Nigeria, whereas the lyophilized preparations retained full activity for at least 6 months.

The complement unit, defined as that amount of complement in the highest dilution of guinea-pig serum which produces 50% haemolysis of an optimally sensitized sheep-cell suspension, was also determined in the preliminary assay between dilutions of guinea-pig serum and dilutions of haemolysin.

Strains of mycoplasma

The strains used and their origins are listed in Table 1; all were preserved at −20°, both frozen in broth culture and lyophilized. The virulence of strains of *Mycoplasma mycoides*, with the exception of strain KH3/J, was confirmed by the subcutaneous inoculation of fresh broth culture into susceptible Zebu bulls. The virulence of strains of *M. capri* was confirmed similarly by subcutaneous inoculation in susceptible goats.

Table 1. *Mycoplasma strains*

Species	Strain	Origin
Mycoplasma mycoides var. *mycoides.*	KH 3/J	Obtained from Khartoum about 1954—preserved lyophilized in Vom at 83rd broth-culture passage—avirulent for local cattle
	MAIDUGURI 8 (M. 8)	Isolated in 1961 in Vom from pneumonic lung received from Bornu Province
	ZAGO	Isolated in 1963 in Vom from pneumonic lung from a natural case of CBPP in Kano Province
	GLADYSDALE (GLAD)	Isolated in Australia in 1953 and stored in frozen tissue—imported in broth culture into Nigeria in 1961
	VICTORIA 5 (V. 5)	Isolated in Australia in 1936. From 10th broth-culture passage, it has been used as a vaccine. Received in Nigeria in 1962 at 21st broth-culture passage—virulent for local cattle
M. mycoides var. *capri*	N/108	Isolated about 1950 from pneumonic goat lung from Benin. Preserved lyophilized at 77th broth-culture passage—virulent for goats. Believed to be the original strain of Longley (1951)
	GOAT 8 (G. 8)	Isolated in 1956 from pneumonic goat lung from Benin—remains virulent for goats
	VOM. I	Isolated in 1962 from pneumonic goat lung from Kano province
	KADUNA I (KAD. I)	Isolated in 1962 from tissue from Kaduna
	Strain 28 (S. 28)	Isolated about 1956 from pneumonic goat lung tissue received from Benin
M. laidlawii	Strain 35 Strain XI Strain XII	Obtained in 1962 as lyophilized cultures from Farcha Laboratory, Republic of Tchad, through the courtesy of Dr A. Provost (Villemot & Provost, 1959*a*)

Antigens

Antigen suspensions of each strain were prepared from ox serum enriched broth cultures as described by Griffin (1964), using a frozen culture as seed. The cultures were centrifuged in a Sharples continuous-flow centrifuge and the organisms harvested in sterile 0·01 M-phosphate buffered saline (PBS, pH 7·3). Untreated mycoplasma CF antigen suspensions (Card, 1959) were used throughout. The crude suspensions were ground in Griffith tubes, washed and resuspended at 1/10 dilution and stored at 4° with thiomersalate (1/10,000) as a preservative.

Antisera

Anti-mycoplasma sera were prepared in New Zealand white rabbits of about 2 kg. bodyweight as described by Griffin (1964). Intravenous injections were used of increasing volumes of washed antigen suspension diluted in PBS to a density equivalent to Brown's opacity tube No. 3 (Burroughs Wellcome). All sera were inactivated at 1/10 dilution by heating for 30 min. in a 56° water bath. Natural haemolysins against sheep red cells were removed by absorbing the sera with sheep red cell stromata. The stromata were prepared by lysing a volume of red cells packed by centrifugation at 1000 *g* for 20 min. with an equal volume of distilled water, followed by freezing and thawing once. The stromata were washed three times in diluent, resuspended to their original volume and stored at −20°. One volume of stromata was mixed with two volumes of rabbit antiserum at 1/10 dilution and the mixture incubated in a water bath at 37° for 20 min. The mixture was centrifuged, the absorbed serum decanted and the absorption process repeated.

Antisera were stored at −20° and working sets in 5·0 ml. volumes to which thiomersalate (1/10,000) was added were dispensed and stored at 4°. Antisera against *Mycoplasma mycoides* strain KH3/J, *M. capri* strain N.108 and *M. laidlawii* strain 35 were designated as representative sera for their particular species.

General procedures for CF tests

Antigen standardization. The object of this assay was to select a routine test dilution of each antigen suspension which in the presence of a 1/10 dilution of an homologous antiserum would fix an arbitrarily chosen amount of complement (1·0 log.-units) under the test conditions. Two-dimensional assays were made in which dilutions of each antigen were titrated against dilutions of guinea-pig serum in the presence of a 1/10 dilution of homologous serum as the constant test component.

Comparison of complement-fixing antigens. The routine test dilution of each mycoplasma antigen, in the presence of dilutions of an homologous or heterologous rabbit anti-mycoplasma serum and dilutions of guinea-pig serum was tested for complement fixation. Strains of each species were examined for antigenic homogeneity before the cross-reactions between species were investigated.

Two-dimensional assays were used throughout in which a log. 0·3 dilution series of each representative antiserum was tested against a log. 0·2 dilution series of guinea-pig serum in the presence of antigen suspension dispensed at the routine test dilution as the constant test component. Two plates were used for each titration; on plate 1 a complete test was done and on plate 2, a serum control plate, the antigen was replaced by unit volume (0·02 ml.) of diluent. A linear complement titration without antigen was included as a control for pro- or anti-complementary activity of the antigen suspension.

The log.-units of complement fixed specifically by each antigen were calculated from the differences in the fixation obtained between corresponding rows in the full test (plate 1) and the antiserum control plate (plate 2). Graphs of the complement fixed (log.-units) by the routine test dilution of each antigen suspension in the presence of antiserum were plotted on graph paper. The areas beneath the curves, which represented the complement fixed, were used as a basis for comparison of the antigens. Areas were measured as described by Kraft & Melnick (1950) with a compensating polar plani-

meter (A. Ott, Kemten, Bayern, West Germany). This method gave the same results as the method of weighing described by Fulton & Dumbell (1949).

RESULTS

Determination of the minimal haemolytic dose of haemolysin and the complement unit

The results of a typical assay are shown in Fig. 1, in which the complement unit was defined in a log. 2·0 dilution of guinea-pig serum. The minimal haemolytic dose of haemolysin was observed at log. 2·7 dilution of the horse anti-sheep red cell serum from which the optimal sensitizing concentration was calculated as log. 2·1 dilution of the particular serum.

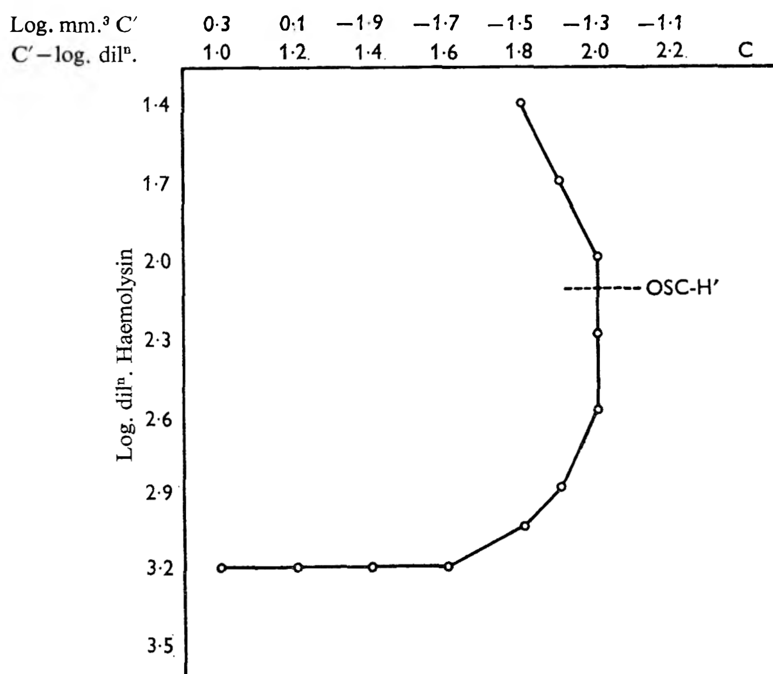

Fig. 1. Plate titration between dilutions of horse anti-sheep haemolysin and dilutions of guinea-pig serum to determine the optimal sensitizing concentration of haemolysin (OSC-H′).

Standardization of antigens

The results of an assay of *Mycoplasma mycoides* strain KH3/J antigen are given in Table 2, and the results for all strains are summarized in Table 3 and shown in Fig. 2, 3, 4, from which the test dilutions of each suspension which fixed 1·0 log.-units (0·2 mm.³) of complement were determined.

Relationship between strains within each species

The results of titrations of dilutions of each representative antiserum and dilutions of guinea-pig serum in the presence of the test dilution of each antigen suspension of the homologous species are shown in Fig. 5, 6, 7. The areas beneath each curve

expressed as fractions of the area obtained in the homologous sets (= 1·0), which enabled direct comparison of the amounts of complement fixed to be made, are shown in Table 4. The results show that strains within each species group were not identical, since reciprocal cross-reactions were not observed. However, within each group substantial cross-reactions did occur, which suggests that the strains are similar antigenically. *Mycoplasma mycoides* strain ZAGO fixed less complement with the representative antiserum, which suggests it was antigenically weak.

Table 2. *Complement fixation of Mycoplasma mycoides strain KH3/J antigen against an homologous rabbit antiserum at 1/10 dilution. Calculation of the amount of complement (C′) fixed*

Antigen dilution (1 in −)	Complement fixed (mm.³ C′)		Specific fixation	
	Antigen + antiserum	Antigen	mm.³ C′	Log_{10} (mm.³ C′)
2	5·00	0·80	4·20	0·62
4	5·00	0·80	4·20	0·62
8	5·00	0·32	4·68	0·67
16	5·00	0·32	4·68	0·67
32	3·40	0·20	3·20	0·51
64	2·00	0·20	1·80	0·25
128	1·00	0·20	0·80	$\bar{1}$·90
256	0·60	0·20	0·40	$\bar{1}$·60
512	0·20	0·20	0	0
0*	0·15	0	0	0

* Antigen dilution replaced by a volume of diluent.

Table 3. *Complement fixed (log. mm.³) by dilutions of Mycoplasma antigens in the presence of homologous rabbit antisera at 1/10 dilution*

Antigen dilution	Complement fixed (log. mm.³)												
	M. mycoides strains					*M. capri* strains					*M. laidlawii* strains		
	KH 3/J	V/5	GLAD.	M. 8	ZAGO	N/108	G. 8	VOM I	S. 28	KAD. I	35	XI	XII
2	0·62	0·62	0·57	0·65	0·62	0·57	0·65	0·65	0·62	0·62	0·62	0·62	0·62
4	0·62	0·65	0·57	0·65	0·62	0·57	0·65	0·65	0·62	0·62	0·62	0·62	0·65
8	0·67	0·65	0·57	0·65	0·43	0·57	0·54	0·57	0·62	0·62	0·62	0·62	0·65
16	0·67	0·54	0·57	0·65	0·18	0·57	0·54	0·46	0·50	0·50	0·50	0·43	0·43
32	0·51	0·43	0·43	0·54	0·18	0·29	0·43	0·23	0·08	0·08	0·08	0·20	0·20
64	0·25	0·30	0·29	0·41	0·02	$\bar{1}$·87	$\bar{1}$·70	$\bar{1}$·97	$\bar{1}$·87	$\bar{1}$·67	$\bar{1}$·87	$\bar{1}$·97	$\bar{1}$·98
128	$\bar{1}$·90	$\bar{1}$·48	$\bar{1}$·87	$\bar{1}$·70	$\bar{1}$·88	$\bar{1}$·70	$\bar{1}$·48	$\bar{1}$·68	$\bar{1}$·67	$\bar{1}$·60	$\bar{1}$·66	$\bar{1}$·88	$\bar{1}$·88
256	$\bar{1}$·60	0	$\bar{1}$·38	$\bar{1}$·48	$\bar{1}$·48	0	0	$\bar{1}$·26	0	0	$\bar{1}$·30	$\bar{1}$·64	$\bar{1}$·70

Relationship between strains of heterologous species

Titrations were made between dilutions of the representative antisera and dilutions of guinea-pig serum in the presence of the test dilutions of each heterologous antigen suspension. The log.-units of complement fixed specifically in each heterologous antigen-antibody reaction were calculated and the results shown graphically (Fig. 8, 9). Areas representing specific complement fixation expressed as fractions of the areas obtained in homologous antigen-antibody sets are included in Table 4.

Cross-reactions of about 25 % identity were observed in tests between *Mycoplasma mycoides* and *M. capri*. *Mycoplasma laidlawii* was judged to be distinct antigenically,

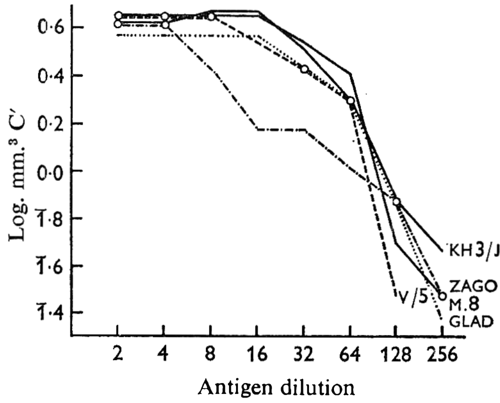

Fig. 2

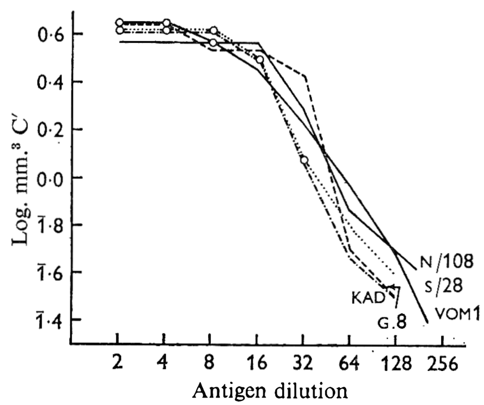

Fig. 3

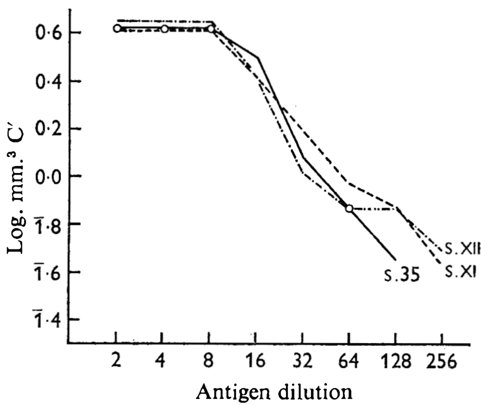

Fig. 4

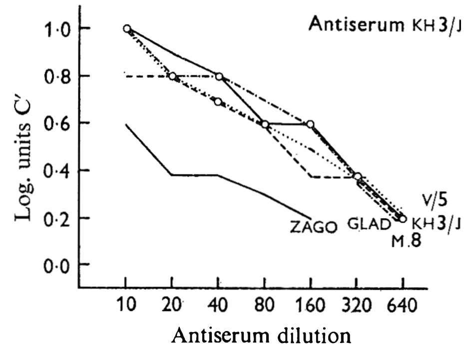

Fig. 5

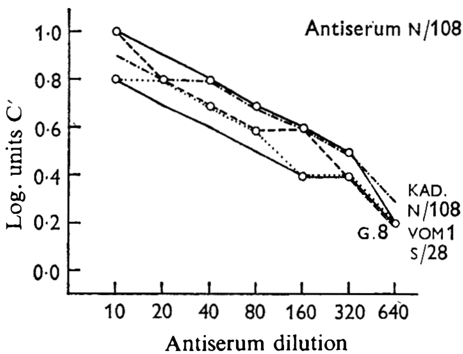

Fig. 6

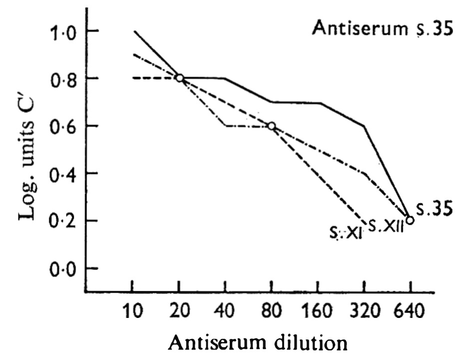

Fig. 7

Fig. 2, 3, 4. Antigen standardization. Complement fixed by dilutions of fresh mycoplasma antigens in the presence of homologous rabbit antisera at 1/10 dilution. Fig. 2. *M. mycoides* antigen suspensions. Fig. 3. *M. capri* antigen suspensions. Fig. 4. *M. laidlawii* antigen suspensions.

Fig. 5, 6, 7. Complement fixed by fresh mycoplasma antigens when titrated against representative antisera of the homologous species. Fig. 5. *M. mycoides* systems. Fig. 6. *M. capri* systems. Fig. 7. *M. laidlawii* systems.

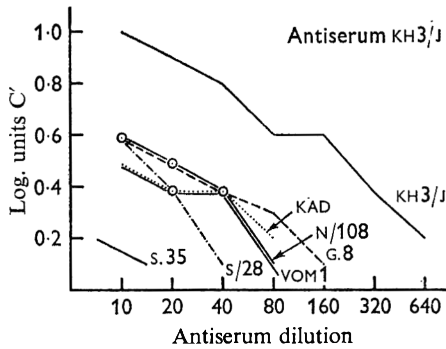

Fig. 8

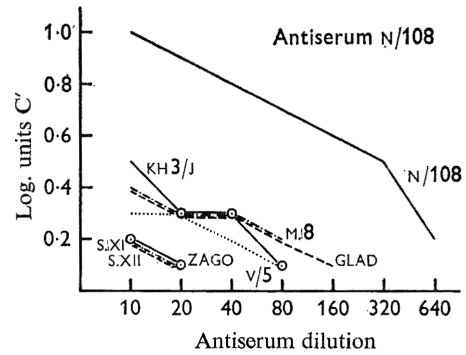

Fig. 9

Fig. 8, 9. Complement fixed by fresh mycoplasma antigens titrated against representative antisera of the heterologous species.

Table 4. *Synoptic table of the antigenic relationships among strains of Mycoplasma mycoides, M. capri and M. laidlawii determined by complement fixation*

	Antiserum								
	M. mycoides			*M. capri*			*M. laidlawii*		
Antigen	KH 3/J	V/5	M. 8	N/108	VOM I	KAD. I	35	XI	XII
M. mycoides									
KH 3/J	1·00*	0·72	0·88	0·22	0·24	0·10	0·007	0	0
V/5	0·88	1·00	0·81	0·12	0·21	0·21	0·014	0	0
M. 8	0·99	0·78	1·00	0·18	0·27	0·21	0·007	0	0
GLAD.	0·79	0·71	0·88	0·19	0·24	0·10	0	0	0
ZAGO	0·24	0·28	0·40	0·015	0·05	0·07	0	0	0
M. capri									
N/108	0·28	0·23	0·19	1·00	0·71	0·69	0	0	0
VOM I	0·25	0·24	0·12	1·00	1·00	0·72	0	0	0
KAD. I	0·23	0·14	0·16	0·82	0·77	1·00	0	0	0
G. 8	0·38	0·34	0·13	0·92	0·86	0·66	0	0	0
S. 28	0·17	0·18	0·13	0·67	0·85	1·00	0	0	0
M. laidlawii									
35	0·015	0	0	0	0	0	1·00	0·46	0·83
XI	0	0·06	0	0·015	0	0·007	0·77	1·00	0·93
XII	0	0·03	0	0·015	0	0·04	0·79	0·83	1·00

* Areas representing specific fixation expressed as proportions of those obtained in homologous antigen-antibody sets (homologous = 1·00).

despite small and irregular cross-reactions with certain strains of *M. mycoides* and *M. capri*. Since reciprocal cross-reactions were not observed, the relationships between *M. mycoides* and *M. capri* have been expressed as the arithmetic means of the complement fixation observed in each complete antigen-antibody set. These results (Table 5) show the size and regularity of the cross-reactions among strains of these species.

Table 5. *Relationship among complement-fixing antigens of strains of Mycoplasma mycoides, M. capri and M. laidlawii. Arithmetic means of the amounts of complement fixed in complete antigen-antibody sets*

	Antiserum								
	M. mycoides			*M. capri*			*M. laidlawii*		
	KH3/J	V/5	M. 8	N/108	VOM I	KAD. I	35	XI	XII
M. mycoides									
KH 3/J	1·00*	0·80	0·94	0·25	0·25	0·17	0·01	0	0
V/5	—	1·00	0·80	0·19	0·20	0·18	0·004	0	0
M. 8	—	—	1·00	0·18	0·28	0·18	0·007	0·03	0·02
M. capri									
N/108	—	—	—	1·00	0·85	0·76	0	0·007	0·007
VOM I	—	—	—	—	1·00	0·75	0	0	0
KAD. I	—	—	—	—	—	1·00	0	0·004	0·12
M. laidlawii									
35	—	—	—	—	—	—	1·00	0·62	0·81
XI	—	—	—	—	—	—	—	1·00	0·89
XII	—	—	—	—	—	—	—	—	1·00

* Arithmetic mean cross-reaction.

DISCUSSION

Mycoplasma laidlawii has been isolated from the bovine genital tract (Edward, Hancock & Hignett, 1947; Villemot & Provost, 1959*a*) and from the bovine respiratory tract (Harbourne, Hunter & Leach, 1965) but its significance is not well understood. Furthermore, because this organism had been shown to be related serologically by both agglutination and precipitation tests to *M. mycoides* (Provost & Villemot, 1959) and to be antigenic for cattle, its possible immunogenicity against contagious bovine pleuropneumonia (CBPP) was suggested (Provost, 1960) although subsequent experiments did not confirm this suggestion (Provost, Villemot & Queval, 1964).

Strains of *Mycoplasma capri*, likewise, related serologically to *M. mycoides* (Provost & Villemot, 1959; Villemot & Provost, 1959*b*) and antigenic for cattle did not protect cattle against CBPP (R. M. Griffin, unpublished data). Hudson (1964), in Australia, also failed in attempts to protect cattle against CBPP with the Y strain of mycoplasma isolated from a goat and related serologically by CF tests to *M. mycoides* (Laws, 1956). *Mycoplasma capri* does not appear to be a naturally occurring pathogen for cattle, although the pathogenicity of strains experimentally have been reported (Heikkilä, 1956; Provost & Villemot, 1959). Opportunities among cattle for transmission of micro-organisms by the respiratory route are frequent because among nomadic cattle-owners in Northern Nigeria it is the practice to graze and kraal together cattle, sheep and goats. It seemed, therefore, among those mycoplasmas which might stimulate in cattle the production of antibodies which cross-react with *M. mycoides*, thereby interfering with the response to serodiagnostic tests for CBPP, that *M. capri* and *M. laidlawii* should first be investigated.

Among the problems found with complement-fixation (CF) techniques for CBPP diagnosis (reviewed by Griffin, 1967), the main difficulty appears to have been the preparation of suitable antigens. The heat-treated, matured suspension of Campbell & Turner (1953) has been preferred generally, but in comparative tests among the

Mycoplasmataceae fresh suspensions have been used recently (Hudson, Cottew & Adler, 1967). Little pro- or anti-complementary activity was found with these suspensions by Stokes (1955); Card (1959) found no difference in potency between fresh and heat-treated antigen suspensions from mycoplasmas of human origin; furthermore, the maturation process did not increase the CF potency.

Comparative CF tests among *Mycoplasma mycoides*, *M. capri* and *M. laidlawii* have given conflicting results. The relationship between *M. mycoides* and *M. capri* was studied by Provost, Villemot, Queval & Borredon (1964) and by Lemcke (1964). They reported results similar to those in the present study, namely, that cross-reactions of about 25% identity were observed. Lemcke found the strains of *M. capri* G1/61, G.11 isolated by Pillai from goats with pleuropneumonia in the Sudan indistinguishable from *M. mycoides*—a result confirmed with strain G1/61 in Australia by Hudson & Cottew (cited by Lemcke, 1964). By contrast, failure to demonstrate a relationship between *M. capri* and *M. mycoides* was reported by Loizelier (1950) and Edward & Leach (1964), who found also that *M. laidlawii* was antigenically distinct.

The relationship between *Mycoplasma mycoides* and strains of *M. laidlawii* of human origin was investigated by Card (1959), who, in linear CF tests between antigen-antibody sets, found a slight cross-reaction. Provost, Villemot, Queval & Borredon (1964), with bovine genital isolates of *M. laidlawii* and antisera prepared in donkeys, obtained results similar to those of Card. They reported also a close relationship between *M. laidlawii* and *M. capri*, a result which conflicts not only with those of the present study in which some of Provost's strains were used, but also with the results of Edward & Leach (1964).

It seems likely that these anomalous results are caused by differences in the cultural methods, antiserum preparation or CF techniques used. Antisera prepared in different hosts and by the use of different injection schedules may stimulate different responses to particular antigens, which may be detected by CF tests. It is difficult to compare the degrees of sensitivity of the many CF techniques which have been used. A refinement which might be applied to comparisons of the amounts of complement fixed would be to define from the complement unit the smallest detectable amount of complement. This could then be used as a base line for graphs used for the measurement of the areas of fixation. Conclusions about these antigenic relationships must await the results of tests with more strains made under standard conditions. However, the antigenic cross-reactions revealed between the strains of *Mycoplasma mycoides* and *M. capri* suggest that this latter organism may be a cause of non-specific positive results in serodiagnostic tests for contagious bovine pleuropneumonia. This postulated relationship needs further field investigation and its significance must be evaluated in the wider context of antigenic cross-reactions among *M. mycoides*, other mycoplasmas, bacteria and polysaccharides from other sources reported recently (Gourlay & Shifrine, 1966; Shifrine & Gourlay, 1967).

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